Invadopodia Formation in Breast Cancer

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I declare that the work presented in this thesis is the work of the author and others' work is fully acknowledged where included

ABSTRACT

Cancer progression can be driven by signalling via growth factors such as hepatocyte growth factor (HGF) and by the surrounding microenvironment. During initial tumour expansion intratumoral hypoxia can develop due to inadequate vasculature supply. To resist the negative effect of hypoxia, cells increase expression of transcription factor hypoxia-inducible-factor-1 (HIF-1α). However, little is known about how HIF-1α might influence the invasive behaviour of the cancer cells. Cell invasion requires actin cytoskeletal reorganisation and cells are thought to utilise specialised membrane protrusions termed invadopodia. Rho family GTPases Rac and Cdc42 through interaction with effector proteins p21-activated kinases (PAKs) are known to regulate actin cytoskeletal dynamics. Indeed, PAK1/2 are over-expressed in breast cancer however the role of PAK1/2 in invadopodia formation has not been fully investigated.

This project sought to investigate the relationship between growth factor signalling, induction of HIF-1α expression and PAK activity during invadopodia formation in MDA-MB-231 cells. Analysis revealed that increased invadopodia formation can be induced by HGF. Furthermore, increasing levels of HIF-1α using dimethyloxaloylglicine (DMOG) treatment or overexpression of GFP-HIF-1α correlated with increased invadopodia activity that was dependent upon the expression of HIF-1α. Subsequent studies revealed that PAK1 and 2 are required for both HGF and DMOG induced invadopodia formation. However, whilst PAK1/2 were activated downstream of HGF, there was no change in PAK1/2 expression levels or activity following DMOG treatment. Interestingly both DMOG treatment and HIF-1α overexpression did lead to up-regulation of cytoskeletal protein expression including β-PIX. β-PIX interacts with PAK1 but has not been previously linked to invadopodia formation. It is shown here for the first time that a reduction in β-PIX expression significantly reduced invadopodia activity following DMOG treatment. Overall, these studies demonstrate the dynamic nature of invadopodia formation; identify novel regulators of invadopodia activity and highlight the direct role of hypoxia in cell invasion.

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ABBREVIATIONS

AID	Autoinhibitory domain
Arf6	ADP-ribosylation factor 6
Asp	Asparagine
BSA	Bovine Serum Albumine
cDNA	DNA complementary to mRNA
CO2	Carbondioxide
DMEM	Dulbecco's Modified Eagles Medium
DMOG	Dimethyloxaloylglycine
DMSO	Dimethylsulfoxide
DNA	Deoxyribose Nucleic Acid
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic Reticulum
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
GBD	GTP-ase binding domain
GEF	Guanine nucleotide exchange factor
GFP	Green Fluorescent Protein
HGF	Hepatocyte growth factor
HIF	Hypoxia Inducible Factor
HRE	Hypoxia Responsive Element
HRP	Horseradish Peroxidase
kDa	Kilodalton
LB	Luria-Bertani Medium
LOX	Lysyl oxidase
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteases
MT1-MMP	Membrane type-1 matrix metalloproteases
NLS	Nuclear Localisation Signal
N-WASP	Neural Wiskott Aldrich syndrome protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHD	Prolyl Hydroxylase Domain Protein
PIX	PAK-interacting exchange factor
pVHL	Von Hippel-Lindau protein
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
shRNA	Short hairpin RNA
siRNA	Short Interfering RNA
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WT	Wildtype

Chapter 1 - Introduction

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1.1 Breast cancer metastasis

Breast cancer is the second most common cause of death in women in western countries (Dumitrescu and Cotarla, 2005; Siegel et al., 2012). One in eight women develop breast cancer at some stage during their life (Jemal et al., 2007) and treatment is particularly difficult when metastasis occurs (Giretti et al., 2008). Diagnosis of advanced breast cancer represents a severe decrease in 5-year survival ~(23.3%) compared with localised forms of the disease ~(98.3%) (Hayat et al., 2007).

The term, metastatic, describes a cancer that has spread to distant organs from the original tumour site. Metastatic breast cancer is the most advanced stage of breast cancer (Hunter et al., 2008) where cancer cells have spread past the breast and axillary (underarm) lymph nodes to other areas of the body, where they continue to grow and proliferate. Breast cancer has the potential to spread to almost any region of the body. The most common region is the bone, followed by the lung and liver. Treatment of metastatic breast cancer generally focuses on relieving symptoms and extending a woman's lifetime. Breast cancer often begins in the breast ducts as ductal carcinoma in situ (DCIS) (Hunter et al., 2008).

In some cases, cancer cells may spread into the bloodstream without being detected in the lymphatic system. In ~10% of breast cancer diagnoses, the cancer cells have already spread to distant organs in the body. A primary diagnosis of Stage IV breast cancer may indicate a rapid progression of the disease or that the cancer was present but not detected in the breast for some time. Despite the recent improvements in survival rates, many patients relapse, and the majority of these patients die because of disseminated metastatic disease, which supports the critical need for new therapeutic strategies.

1.2 Metastatic cascade

Cancer metastasis involves a series of changes in cell behaviour, driven by oncogenic transformation, which leads to local invasion of the basement membrane and surrounding tissue, migration through tissue, entry or intravasation into the blood vessels (vascular) or lymphatic system and survival there, followed by extravasation/colonization and growth at different organ sites **(figure 1.1)** (Siegel et al., 2011; Sporn, 1996)**.** To achieve these steps, precise coordination of cell movement and matrix remodelling are essential (Friedl and Wolf, 2003). Each of these steps requires a recognised molecular program in which the regulation of the adhesive, migratory and cytoskeletal properties of the spreading tumour cells play pivotal roles (Yilmaz and Christofori, 2009). At any given time, only a small proportion of tumour cells are invading and disseminating (Wang et al., 2004). Understanding the mechanisms that drive invasive migration of these tumour cells is crucial to better understand metastasis.

The acquisition of an invasive phenotype is a hallmark of metastatic cancer and signifies a poorer prognosis (Hanahan and Weinberg, 2000). One of the key aspects of tumour metastasis is that tumour cells transform from local, non-invasive confined cells to migrating, metastatic cancer cells. This transition process is initiated when the cells obtain the ability to dissociate from intercellular adhesions and become motile (Friedl and Wolf, 2003), which is usually driven by complex regulatory signalling cascades that transiently and/or permanently alter the expression of multiple proteins that act to reorganize the cytoskeletal network (Kedrin et al., 2007; Yamazaki et al., 2005).

Invasion into the surrounding stromal tissues requires the coordinated regulation of both actin cytoskeletal rearrangement and cell substratum adhesion turnover (Chaffer and Weinberg, 2011; Chambers et al., 2002) **(figure 1.1).** To successfully migrate through the stromal microenvironment, cells must be able to extend processes (lamellipodia, filopodia, invadopodia), anchor these nascent protrusions to the underlying matrix (cell adhesions), generate the forces required for forward movement and ultimately dissolve adhesions at the rear of the cell (Friedl and Alexander, 2011; Nurnberg et al., 2011).

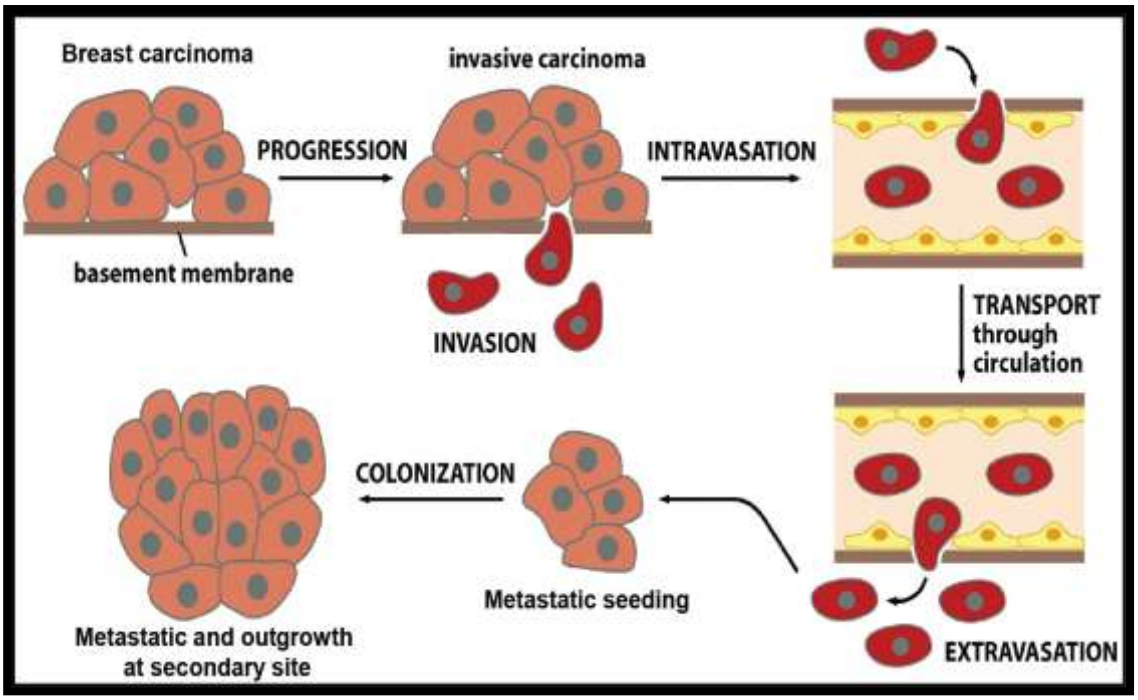

Figure 1.1 Progression of metastatic cancer. Schematic representation of epithelial carcinoma as a multistage process beginning with oncogenic transformation of cells, aberrant growth and proliferation to form a primary tumour. Tumour cells eventually acquire a migratory phenotype and invade their surrounding extracellular matrix (ECM) in a process possibly involving interplay with non-tumour cells such as fibroblasts. Cells metastasise, entering blood or lymphatic vessels by intravasation. At some point, metastatic cancer cells will attach and extravasate, establishing a secondary site.

Invasion is a key ability of cells in a variety of both physiological and pathological scenarios, including migration of immune cells to sites of infection or dissemination of cancer cells during metastasis. In all cases, cells encounter barriers hindering their mobility such as blood vessel walls, tissue boundaries and more importantly, the meshwork of the extracellular matrix (ECM). This complex network of fibrillar proteins and polysaccharides bridges the intercellular space, where it functions as a surface for cell adhesion molecules and supports tissue connectivity and differentiation (Hynes, 2009). Cancer cell invasion involves rapid production of extracellular proteases which act to degrade the ECM (Deryugina and Quigley, 2011).

Depending on the meshwork size of the ECM, cells are either able to undergo amoeboid, non-lytic invasion through interfibrillar gaps or are forced to proteolytically degrade denser matrix material (Friedl and Wolf, 2003). Recent evidence indicates that, *in vivo*, covalent cross-links are typically present between ECM fibers, which would favour proteolytic mechanisms of invasion (Sabeh et al., 2009). Therefore, the mechanisms of localised ECM degradation are likely of central importance for cell invasion in both health and disease.

1.3 Promotion of cancer cell metastasis by environmental factors

Migration and invasion of cells in tumour stroma result from co-operation among protrusive, adhesive, contractile, and proteolytic mechanisms. These occur as a result of the combined influence that both tumour and stromal cells have on the content and architecture of the ECM, as well as on the autocrine and paracrine production and activation of proteinases and growth factors (Hanahan and Weinberg, 2000). The aberrant proliferation of cells harbouring oncogenic lesions is challenged by multiple layers of mechanisms that affect tumour formation. Whilst several of these barriers are intrinsic to the cell, an entirely distinct class of pressures comes from sources that are extrinsic to the cancerous cells. Factors in the tumour microenvironment that influence tumour progression includes growth factors and the limited availability of nutrients and oxygen. How tumour cells respond to these external cues influences, sometimes in dramatic fashion, their metastatic potential (Fidler, 2003).

1.3.1 Growth factors

Extrinsic factors in the tumour microenvironment can promote the invasion of cancer cells. Indeed, micro-metastases are often genetically heterogeneous, suggesting that invasive behaviour might not be entirely intrinsically specified (Klein et al., 2002). Growth factor signalling can modulate invasive activity either directly or indirectly. The complex microenvironment surrounding tumour cells contains migration-stimulating factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) produced by tumour cells themselves; matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) produced by fibroblasts and endothelial cells, transforming growth factor-β (TGF-β)

also produced by tumour cells or released from the ECM by proteinase activity and platelet-derived growth factor (PDGF) produced by tumour cells (Liotta and Kohn, 2001).

Tumour-associated macrophages express epidermal growth factor (EGF) and promote EGF receptor-dependent carcinoma cell invasion *in vivo* (Wyckoff et al., 2004). Accumulating evidence suggests that EGF receptor ligands and the EGF receptor signalling pathway are critical for the invasiveness and metastasis of cancer cells. Furthermore, invasive carcinoma cells and tumour-associated macrophages have been shown to interact through the CSF-1/EGF paracrine loop, which enhance migration, invasion and intravasation of carcinoma cells (Goswami et al., 2005; Wyckoff et al., 2004).

Myofibroblasts can promote 'mesenchymal' cancer cell invasion by producing hepatocyte growth factor (HGF)/scatter factor (SF), which is the ligand for the c-Met receptor (De Wever et al., 2004). Whilst HGF/SF-induced cellular effects are required for many normal physiological processes, aberrant Met activation has been implicated in most types of solid tumours, often correlating with poor prognosis. In this case, activation of c-Met leads to an invasion process by which tumour cells weaken tissue constraints, migrate and colonise foreign districts resulting in metastasis (Birchmeier et al., 1997). More specifically, the exposure of Met-expressing cells to HGF/SF can elicit a variety of cellular responses including proliferation, migration, invasion and branching morphogenesis (Birchmeier et al., 2003; Gao and Woude, 2005).

1.3.2 Hypoxia

Breast epithelial cells, similar to other epithelia, are able to survive and differentiate because of the tissue architecture and the growth factor milieu present in the mammary gland (Hagios et al., 1998). This rich environment, however, is progressively lost during malignant transformation, especially as malignant cells become invasive and metastatic. Such cells must acquire mechanisms that maintain their survival and promote their invasion outside the confines of the mammary gland (Fearon, 1999; Hanahan and Weinberg, 2000). Alongside growth factors in the

tumour microenvironment, tumour cells must survive under environmental stresses not present in normal tissue. One of the most formidable barriers to their survival is hypoxia (Brown, 1999), which is emerging as a major factor that shapes the aggressiveness of breast tumours. The oxygen tension within many solid tumours is substantially less than that in the adjacent normal tissue usually with pO_2 values less than 5-10mmHg (Brown, 1999; Brown and William, 2004; Travers, 2006).

This phenomenon is due to poor vascularisation during tumour expansion (Brown, 1999; Hockel and Vaupel, 2001). Although hypoxia can destroy most normal cells and some tumour cells, it also provides a strong selective pressure for the survival of the most aggressive and metastatic cells. In fact, clinical studies have clearly shown that the low pO_2 tension within a neoplastic lesion is an independent prognostic indicator of poor outcome and correlates with an increased risk to develop distant metastasis irrespective of therapeutic treatment (Brown, 1999; Hockel and Vaupel, 2001). Although a limiting factor for tumour growth, hypoxia alters important intracellular pathways and is recognised as a positive stimulus for invasion, angiogenesis and metastasis (Semenza, 2002). Tumour cells unlike normal cells, are able to survive adverse hypoxic conditions through a diversity of mechanisms (Reynolds et al., 1996; Semenza, 2003; Xie and Huang, 2003) including changes in gene expression, inactivation and/or activation of genes and enhanced genomic instability (Denko et al., 2003; Lal et al., 2001; Semenza, 2000; Semenza, 2006; Vaupel, 2004). Cells that are able to resist the negative effects of low oxygen conditions and then competitively flourish in hypoxic environments have been shown to be highly dependent on the expression of the transcription factor hypoxia-inducible-factor-1 (HIF-1) (Semenza and Wang, 1992). HIF is activated in low oxygen (1% O_2) and coordinates extensive changes in transcription, which leads to broadly adaptive changes, including increased erythropoiesis, glycolysis, and angiogenesis (Maxwell, 2005). HIF-1 was discovered in 1992 when the mechanisms of hypoxia that induce erythropoietin expression were dissected (Semenza and Wang, 1992). One of the best characterised HIF-dependent genes is the potent endothelial mitogen, VEGF, which regulates endothelial cell proliferation and blood vessel formation in both normal and cancerous tissues (Liu et al., 1995).

Approximately 40% of breast carcinomas examined were found to contain hypoxic tissue areas (pO_2 of <2.5mmHg) (Vaupel et al., 1991). Up-regulation of HIF-1α signalling pathways have been associated with the molecular expression signature of micro metastasis in human breast cancer (Woelfle et al., 2003). HIF-1α is over-expressed in most common human cancers, including breast, brain, cervical, colon, lung, ovarian and prostate (Semenza, 2002) and has been associated with increased blood vessel density, tumour aggressiveness and poor prognosis (Vleugel et al., 2005). In early stage cervical carcinoma, HIF-1α over-expression is correlated with patient mortality (Birner et al., 2000). Also, during early stages of esophageal cancer, HIF-1α over-expression is associated with failure to achieve a complete response to radiotherapy (Koukourakis et al., 2001).

1.3.3 HIF-1 structure

HIF-1 is a heterodimeric protein that is composed of an O_2-dependent 120 kDa HIF-1α subunit and a 91-94 kDa constitutively expressed aryl hydrocarbon (Ah) nuclear receptor translocator (ARNT), also known as HIF-1β (Wang, 1995). Both polypeptides belong to the basic helix-loop-helix (bHLH)-PAS family (PAS is an acronym for the three members first recognised: Per, ARNT, Sim). In HIF-1α and HIF-1β, the N-terminal bHLH domain precedes the PAS domains **(figure 1.2)**. The basic domain is essential for DNA binding, whereas the HLH and the PAS domains are necessary for hetero-dimerization. Both polypeptides contain nuclear localization signals (NLS). Functionally important are two transcriptional activation domains (TAD) in HIF-1α, an N-terminal TAD (N-TAD) that partially overlaps with the oxygen-dependent degradation domain (ODDD) and a C-terminal TAD (C-TAD)important for cofactor recruitment **(figure 1.2).** HIF-1α belongs to a family of related proteins including HIF-2α (Tian et al., 1997) and HIF-3α (Gu et al., 1998). While both HIF-2α and HIF-3α show similar biochemical properties such as hetero-dimerization with HIF-1β, their biological functions remain unclear. HIF-2α has extensive sequence similarity to HIF-1α; it is also regulated by proline and asparagine hydroxylation, dimerizes with HIF-1β and activates transcription of a group of target genes that overlaps with, but is distinct from those regulated by HIF-1α (Lau et al., 2007). In contrast, it has been proposed that HIF-3α is an inhibitor of

HIF-1 that may be involved in feedback regulation because its expression is transcriptionally regulated by HIF-1 (Makino et al., 2007)

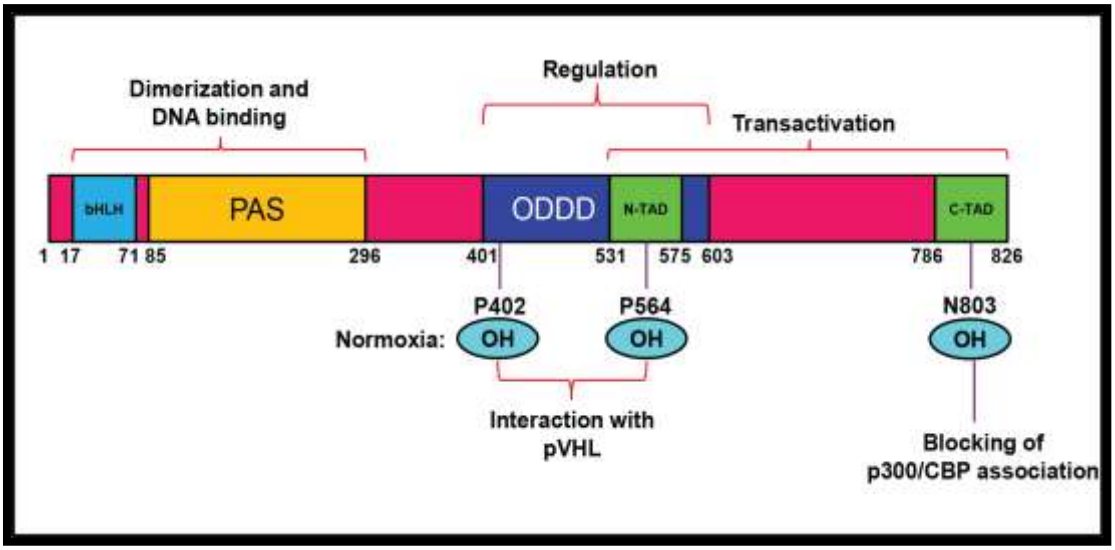

Figure 1.2 Schematic representation of HIF-1α and its four functional domains. The basic-helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domains of hypoxia-inducible factor-1α (HIF-1α) are involved in dimerization and DNA binding; the oxygen-dependent degradation domain (ODDD) is required for degradation via the proteasome; and the transactivation domains (N-TAD and C-TAD) are involved in transactivation activity. Hydroxylation of proline residues 402 and 564 within the ODDD mediates its interaction with the von Hippel-Lindau tumour suppressor protein (pVHL). Hydroxylation of asparagine 803 in the C-TAD blocks its association with transcriptional co-activator p300/CBP. Adapted from (Poon et al., 2009)

1.3.4 HIF-1 Regulation

HIF-1 activity is primarily regulated by the abundance of the α-subunit. Although the HIF-1α gene is constitutively transcribed, HIF-1α is highly unstable and virtually undetectable at normal oxygen levels (normoxic condition 20% O_2) due to its oxygen dependent degradation. In contrast, HIF-1β is constitutively found in the nucleus of all cells (Wang, 1995). HIF-1α is subjected to O_2-dependent hydroxylation on proline residue 402 and 564 by prolyl hydroxylase domain protein 2 (PHD2). Both residues are situated in the oxygen-dependent degradation domain (ODDD) of HIF-1α **(figure 1.3)**. Three different HIF prolyl-hydroxylases have been described, termed PHD1, PHD2, and PHD3 by way of their different prolyl hydroxylase domain (Bruick and McKnight, 2001; Epstein et al., 2001). Although all three PHDs have been shown to regulate HIF-1α, the key isoform responsible for HIF-1α

regulation in many cell types is PHD2 (Appelhoff et al., 2004; Berra et al., 2003). It has been shown that PHD1 and PHD3 also hydroxylate HIF-1α in *vivo* and *in vitro*. However, the exact relevance of these two isoforms in cells remains to be elucidated (Appelhoff et al., 2004; Epstein et al., 2001). PHD enzymes hydroxylate HIF-1α using oxygen and 2-oxoglutarate as substrates and iron and ascorbate as essential cofactors **(figure 1.3).** This modification creates an interface for interaction with the von Hippel-Lindau tumour suppressor protein (VHL), which recruits an E3 ubiquitin-protein ligase that catalyzes polyubiquitination of HIF-1α, thereby targeting it for proteosomal degradation (Kaelin and Ratcliffe, 2008).

Under hypoxic conditions (~1% O_2), the major substrate dioxygen is unavailable and thus hydroxylation is inhibited and HIF-1α can escape degradation, instead it rapidly accumulates and translocates into the nucleus to dimerize with HIF-1β **(figure 1.3)**. The heterodimeric HIF-1 complex binds to the conserved DNA binding sequence, 5'-RCGTG-3' (where R is either an A or G), in the hypoxia response element (HRE) on the promoter region of target genes. HIF activates the transcription of its target genes such as the proangiogenic growth factor, VEGF, heme oxygenase, glucose transporter-1 (GLUT1), and erythropoietin (EPO) (Semenza and Wang, 1992; Wang, 1995) by recruiting additional coactivators/cofactors such as p300, CREB binding protein (CBP) and steroid receptor coactivator (SRC)-1.

HIF-1α activity is also controlled by O_2-dependent hydroxylation of asparagine-803 in the C-TAD of HIF-1α. This is mediated by the asparagine hydroxylase factor inhibiting HIF-1 (FIH-1) which acts by blocking the interaction of HIF-1α with coactivators p300 and CBP under normoxic conditions (Lando et al., 2002). Both PHD2 and FIH-1 utilize O_2 and α-ketoglutarate as substrates and generate CO_2 and succinate as by-products of the hydroxylation reaction. Additionally, Siah-1 (a ubiquitin ligase) has been identified as facilitating proteosomal FIH degradation under hypoxic conditions (Fukuba et al., 2007).

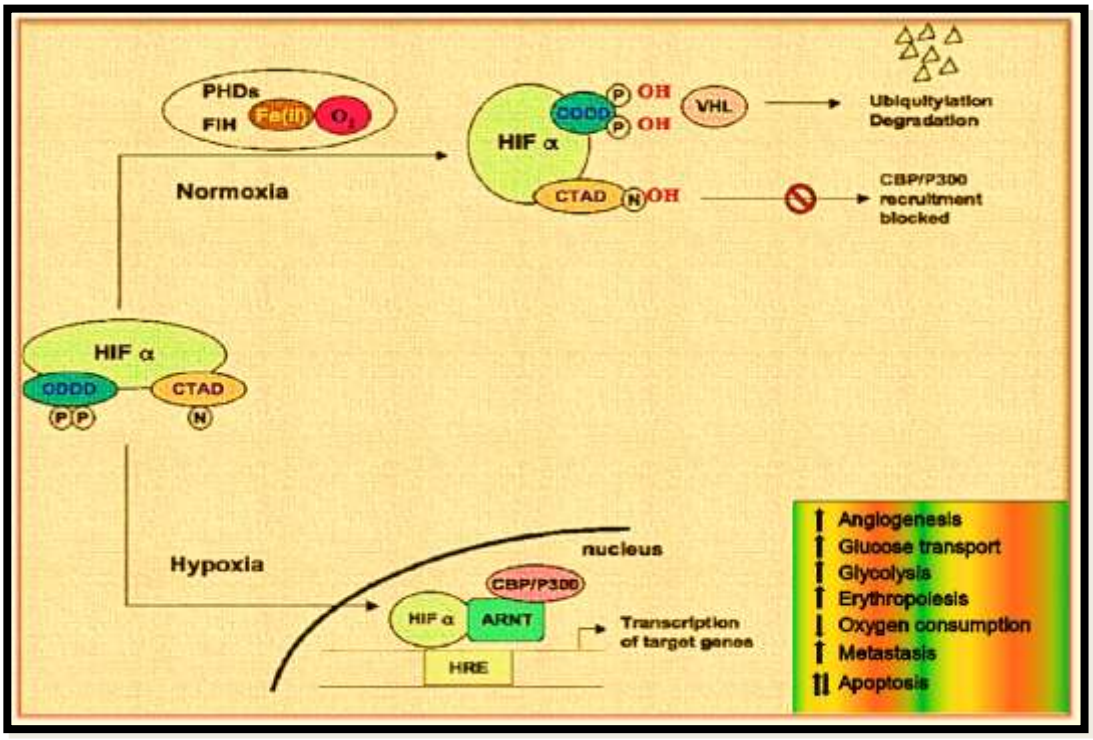

Figure 1.3 Regulation of HIF-α during normoxia and hypoxia. In the presence of oxygen, HIF-α is hydroxylated (OH) at two proline residues (P) and one asparaginyl residue (N). These lie within the oxygen dependent degradation domain (ODDD) and C-terminal transactivation domain (CTAD) respectively. The prolyl hydroxylation leads to capture by von Hippel-Lindau tumour suppressor (VHL). The asparaginyl hydroxylation blocks co-activator recruitment. This results in HIF destruction. In the absence of hydroxylation under hypoxic conditions, HIF-α moves to the nucleus where it heterodimerises with HIF-1β/ARNT and the CTAD recruits the co-activator CBP/P300 and activates expression of target genes. Adapted from (Kiriakidis et al., 2007).

Members of the CITED (CBP/p300 interacting transactivator with ED-rich tail) family, such as CITED2 (Bhattacharya et al., 1999) and CITED4 (Fox et al., 2004), were found to bind to p300. This binding disrupts the interaction between p300 and HIF-1α, thus inhibiting HIF-1 transactivation and gene expression during hypoxia. An alternative splicing product of the HIF-3α locus named inhibitory PAS protein (IPAS) has been found to function as a dominant negative regulator of HIF function (Makino et al., 2001). IPAS complexes with HIF proteins and thereby impairs productive interaction between HIF and hypoxia response elements of target genes. This inhibitory protein lacks the transactivation domain and is expressed mainly in corneal epithelium, retina and brain tissue.

The levels of HIF-1α expression and activity are also controlled by major signal transduction pathways, including those involving phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase (MAPK/ERK). Under normoxic conditions, growth factors and cytokines induce PI3K and its downstream effectors Akt, mTOR and p70S6K, leading to increased HIF-1α levels (Bardos and Athcroft, 2004). Activation of Src, which lies downstream of receptor tyrosine kinases and upstream of the PI3K pathway, also induces HIF-1α expression (Jiang et al., 1997). Yet, the mechanism by which the PI3K pathway potentiates HIF-1 activity under hypoxic conditions remains unclear and moreover, it is not known whether GSK3β, a downstream target of Akt, plays a role. However, under prolonged hypoxia it may be that GSK3β prevents the accumulation of HIF-1α (Schnitzer et al., 2005). ERK1/2 MAP kinases have been shown to phosphorylate HIF-1α at specific sites and are mainly thought to be implicated in the nuclear accumulation and transcriptional activity of HIF-1α (Sang et al., 2003).

1.3.5 HIF-1α in cancer cell invasion

HIF-1α is involved in tumour angiogenesis and metastasis by regulating genes involved in the response to hypoxia. Clinical evidence showed that HIF-1α is associated with a worse prognosis in patients with invasive breast carcinoma (Dales et al., 2005). An important property of tumour cells that are able to survive under hypoxia is that they exhibit an enhanced propensity to invade (Le et al., 2004), a finding that underscores the hypothesis that hypoxia facilitates tumour progression. An understanding of the mechanism by which hypoxia stimulates invasion would provide insight into the nature of invasion itself. HIF-1 acts as a master regulator of oxygen-regulated gene expression. Four groups of direct HIF-1 target genes particularly relevant to cancer, encode angiogenic factors, proliferation/survival factors, glucose transporters/glycolytic enzymes and invasion factors (Semenza, 2003). The products of the genes that HIF-1 regulates act at several steps in the invasive migration processes. Invasion of the basement membrane is the defining characteristic of epithelial cancers. Epithelial cells are normally constrained by cell-cell contacts and by the basement membrane. Basement membrane disruption, through a proteolytic cascade (Krishnamachary et al., 2003) which digests the basement membrane/ECM, was shown to be promoted by HIF-1α-dependent up-regulation of cathepsin D (CTSD), proteases including uPAR and matrix

metalloproteinase-2 (MMP2). Subsequently as a result of hypoxia, degraded ECM is replaced by the production of fibronectin-rich matrix and other ECM proteins that are recognised by integrins expressed on the cancer cell surface (Krishnamachary et al., 2003). Hypoxia-induced HIF-1α also up-regulates expression of lysyl oxidase (LOX), an extracellular enzyme that covalently modifies collagens to increase focal adhesion kinase (FAK) activity, cell migration and metastasis (Erler et al., 2006). Another mechanism inducing increased invasive migration is through the activation or over-expression of surface tyrosine kinase c-Met receptors by HIF-1α (Pennacchietti et al., 2003). Following c-Met over-expression, tumour cells can then respond to HGF, which is produced at an elevated level, under hypoxia (Ide et al., 2006) .

HIF-1α protein has been suggested to be a key factor in glioma cell migration and invasion under hypoxic conditions. It has also been reported that chondrosarcoma cell invasion is increased by hypoxia induced expression of CXCR4 and MMP1 and is mediated by HIF-1α and ERK (Sun et al., 2010). Recently, Ras was shown to be activated in response to hypoxia, which then aids stabilization of HIF-1α, and in turn upregulates MMP-9 expression (Choi et al., 2011). MMP-9 is upregulated concurrently with HIF-1α in tumour tissues from patients with breast cancer suggesting that HIF-1α promotes cell invasion through an MMP-9-dependent mechanism (Choi et al., 2011).

Rho GTPases have been implicated in oxygen sensing since Rac1 was identified as a component of the NADPH oxidase complex in phagocytes (Abo et al., 1992). Subsequent studies demonstrate that Cdc42 activity is required for hypoxia-induced activation of HIF-1α in renal carcinoma cells (Turcotte et al., 2003). Small GTPases are involved in the motility and invasion of a variety of carcinoma cell lines and have emerged as important control elements in the reorganization of the actin cytoskeleton and vesicular trafficking (Oxford and Theodorescu, 2003). Hypoxia was shown to induce activation of RhoA in breast carcinoma as early as 6 hours after hypoxia exposure (Munoz-Najar et al., 2006). Additionally, dominant-negative expression of RhoA specifically blocked hypoxia-induced invasion and plasma membrane localization of MT1-MMP (Munoz-Najar et al., 2006) confirming a role for this GTPase in the cellular adaptation to hypoxia.

Recently, over-expression of RhoE in normoxia or hypoxia-induced HIF-1α expression was shown to up-regulate the mesencymal marker Vimentin, down-regulate the epithelial marker E-cadherin, and significantly increase cell invasion in vitro (Zhou et al., 2011). In gastric cancer cells, RhoE was identified as a direct target of HIF-1 and extopic expression of the Rho GTPase in these cells induced the up-regulation of MMP2/MMP-9. Therefore, RhoE up-regulation represents a pivotal cellular adaptive response to hypoxia with implications in gastric cancer cell invasion (Zhou et al., 2011).

Rac1 has been demonstrated as an intermediate in the PI3K-mediated induction of HIF-1α (Xue et al., 2011). There was a significant down regulation of the tumour suppressor genes p53 and VHL in cells expressing a constitutively active form of Rac1. Rac1-mediated inhibition of p53 and VHL could therefore be causal in the up regulation of HIF-1α expression (Xue et al., 2011). Hypoxia was shown to stimulate cell invasion by stabilizing microtubules and promoting α6β4 integrin trafficking to the plasma membrane (Yoon et al., 2005).

Since this study was initiated, one report has suggested that hypoxia promotes invadopodia formation in human fibrosarcoma cells (Lucien et al., 2011). Hypoxia increases the activity of NHE-1 (a pH-regulator in cancer cells) which occurs in a HIF-1 independent manner and NHE-1 was found to be required for hypoxia-induced invadopodia formation and function in cancer cells (Lucien et al., 2011). However, so far, there is no evidence to show a direct effect of increased HIF-1α expression on invadopodia formation and matrix degradation in breast carcinoma cells.

1.4 Invadopodia as a marker of invasive potential

Invasion of cells through layers of extracellular matrix is a key step in tumour metastasis (Weaver, 2006). Stages of invasion include adhesion to the matrix, degradation of proximal matrix molecules, extension and traction of the cell on the newly revealed matrix, and movement of the cell body through the resulting gap in the matrix. To become invasive, the first barrier that tumour cells must overcome is

their epithelial basement-membrane**.** In order to cross the basement membrane, tumour cells are thought to require specialised membrane protrusions in the direction of cell movement. These structures are rich in actin filaments, integrins, tyrosine kinase signalling machinery, adhesion proteins, membrane-type and secreted proteases (MMPs) and signalling proteins that regulate the actin cytoskeleton and membrane remodelling. These protrusions are termed invadopodia. There is growing evidence that the formation of invadopodia is part of the invasion process (Eckert et al., 2011; Yilmaz and Christofori, 2009). It is currently unclear the degree to which carcinoma cells must proteolytically digest the epithelial basement membrane as they initially invade, and whether stromal cells are also involved in this process. However, recently there is evidence for the involvement of invadopodia-mediated proteolysis of vascular basement membrane during intravasation and metastasis (Gligorijevic et al., 2012). Localised proteolytic activity of invasive cells has been demonstrated when these cells are cultured on different substrates such as gelatin, fibronectin, collagen type I, collagen type IV or laminin (Chen, 1989; Kelly et al., 1994; Mueller and Chen, 1991).

1.4.1 Discovery

Invadopodia were discovered in Rous sarcoma virus-transformed fibroblasts, driven by oncogenic vSrc tyrosine kinase (Chen, 1989). Src-transformed cells grown on fibronectin formed prominent ventral protrusions with adhesive (Tarone et al., 1985) and degradative properties (Chen et al., 1984). In addition, vSrc was found to localise at the sites of fibronectin degradation (Chen et al., 1985). Invadopodia were subsequently described in invasive tumour cells such as human malignant breast tumour cells MDA-MB-231 (Chen et al., 1994) and human malignant melanoma cells (Aoyama and Chen, 1990). Moreover, these specialised structures have been identified in cell lines or primary tumour cells from malignant melanoma (Baldassarre et al., 2003; Mueller et al., 1999; Seals et al., 2005; Tague et al., 2004), breast/mammary carcinoma (Artym et al., 2006; Hashimoto et al., 2004; Lorenz et al., 2004), glioma (Angers-Loustau et al., 2004; Chuang et al., 2004), head and neck squamous cell carcinoma (Clark et al., 2007) and prostate carcinoma cells (Desai et al., 2008). Invadopodia are distinct from other cell adherence sites such as focal contacts and focal adhesions. Indeed, these adhesions are not traditionally associated

with proteolytic activity (Chen, 1989). However, a recent study has shown that focal adhesions can also promote matrix degradation activity in highly invasive cells (Wang and McNiven, 2012).

1.4.2 Molecular characteristics of invadopodia

At the ultra-structural level, invadopodia are filament-like extensions protruding from the ventral surface of cells adherent to matrix **(figure 1.4)**. The size of invadopodia ranges from 8-10 µm in diameter and reach up to 5 µm in depth (Linder, 2009). Time-lapse image analysis has shown that invadopodia are formed *de novo* and their lifetime varies from minutes to several hours. An *in vitro* matrix degradation assay is used to detect invadopodia activity (Artym et al., 2006; Chen et al., 1984; Yamaguchi et al., 2006). In this assay, cells are seeded on two-dimensional (2D) surfaces coated with matrix proteins, and form invadopodia at the ventral surface. Routinely, the matrix is fluorescently-labelled native ECM substrates for example fibronectin or with substitute proteins such as gelatin (denatured collagen) **(figure 1.4).** Digestion of matrices by invadopodia at discrete sites under the cells results in fluorescence-negative cavities with dot-like accumulations of F-actin which are detected by fluorescence microscopy (Artym et al., 2009). Recently, a study demonstrated that cells assemble invadopodia-like structures and invade into matrigel in a circular invasion assay (Yu and Machesky, 2012). This assay is proposed to be useful for comparison of cell migration parameters with cell invasion and for visualizing cells whilst they interact with a 3D matrix but still remain close to a glass surface.

Invadopodia are often highly enriched with actin filaments (F-actin) and components required for actin assembly including the Arp2/3 actin-nucleating complex, neural Wiskott Aldrich Syndrome protein (N-WASP) and cortactin (Artym et al., 2006; Buccione et al., 2004; Clark et al., 2007; Linder, 2007; Lorenz et al., 2004; Mizutani et al., 2002). Tyrosine phosphorylation of cortactin causes its dissociation from cofilin enabling cofilin to generate barbed ends by severing actin filaments and thus initiating actin polymerization (Oser and Condeelis, 2009; Oser et al., 2009). After barbed end formation, cortactin is dephosphorylated, which blocks cofilin severing activity thereby stabilizing invadopodia and the maturation of invadopodia (Oser et

al., 2009; Yamaguchi et al., 2005). Live cell kinetic studies have identified four distinct phases of invadopodia maturation on the basis of i) localisation of cortactin to ventral actin puncta, ii) the recruitment of membrane type-1 matrix metalloproteinases (MT1-MMP) to these sites, iii) the appearance of degraded foci in the underlying matrix, and iv) the dissociation of cortactin from these sites (Artym et al., 2006).

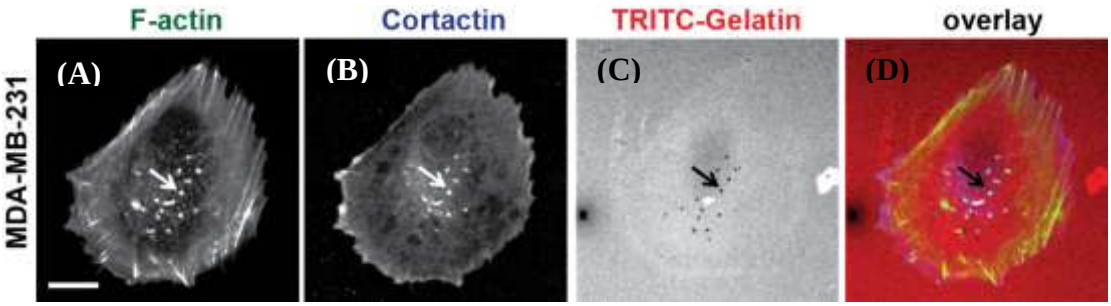

Figure 1.4 Confocal microscopy image of double immunofluorescence labelling of MDA-MB-231 carcinoma cells plated on TRITC-conjugated gelatin: (A) invadopodia structures marked by phalloidin-488; (B) invadopodia structures marked by anti-cortactin antibody; (C) degradation areas on the underlying TRITC-conjugated gelatin. Arrows indicate the colocalization between actin, cortactin and puncta of degraded extracellular matrix (ECM).

Other features that can be used to recognize invadopodia in some cell lines, include their localisation close to the nucleus and proximal to the Golgi complex, the central regulator for intracellular trafficking (Baldassarre et al., 2003), and their extended half-life of up to two hours or more (Baldassarre et al., 2006; Yamaguchi et al., 2005) in comparison to other protrusive adhesions. Cell culture in 3D that mimics the *in vivo* environment has been used to reveal differences in the morphology, metabolism and survival of cancer cells (Murphy and Courtneidge, 2011). There is much circumstantial evidence to suggest that invadopodia are physiologically relevant structures. Recent studies have used 3D systems to address invadopodia formation and function, by which invading cells were thought to frequently extend long protrusions that degrade the matrix (Friedl and Gilmour, 2009; Schoumacher et al., 2010; Wolf and Friedl, 2009).

1.5 Induction of invadopodia formation

Studies have shown that stimulation with growth factors, such as platelet-derived growth factor (PDGF) (Eckert et al., 2011) and transforming growth factor-β (TGFβ) (Rottiers et al., 2009) induces invadopodia in cancer cells and podosome formation in normal cells respectively. These stimuli elicit the phosphorylation and/or activation of the key invadopodia-associated proteins through canonical signalling pathways.

1.5.1 Stimulation by EGF/HGF

EGF is a well-known growth factor that induces dynamic cell protrusions associated with the actin cytoskeleton. EGF and CSF-1 stimulation enhances invadopodia formation and ECM degradation in metastatic rat MTLn3 mammary carcinoma cells and podosomes in macrophages, respectively (Yamaguchi et al., 2005; Yamaguchi et al., 2006). Recently, EGF has also been implicated in the induction of invadopodia formation in human breast carcinoma cells (Mader et al., 2011). However, this work is still preliminary and has not been validated across several human cell lines from other tissue types.

In LOX melanoma cells, HGF stimulation of endogenous c-Met is implicated in enhancing matrix substrate degradation, possibly via induction of invadopodia activity (Tague et al., 2004). This effect of HGF was completely suppressed by a dominant negative mutant of small GTPase, ADP-ribosylation factor 6 (Arf6) (Tague et al., 2004). Recently, a report has suggested that HGF promotes invadopodia formation in breast carcinoma cells and that this formation is dependent on Met activity (Rajadurai et al., 2012). Furthermore, Met localizes to invadopodia along with cortactin and promotes phosphorylation of cortactin (Rajadurai et al., 2012).

1.5.2 Rho family GTPases

Invadopodia formation requires reorganization of the actin cytoskeleton. The Rho family small G proteins, consisting of the Rho, Rac and Cdc42 subfamilies, are implicated in various cell functions, such as cell shape change, adhesion, motility,

and invasion through their ability to coordinate reorganization of the actin cytoskeleton (Hall, 1998; Takai et al., 1995).

Rho, Rac and Cdc42 are the most studied Rho family GTPases, these proteins act as molecular switches existing in two conformational states, inactive-GDP and active-GTP bound (Jaffe and Hall, 2005). Only in a GTP-bound state are these proteins able to bind effector proteins and transfer signals from a pool of membrane receptors such as cytokine and growth factor, adhesion and G-protein receptors (Buchsbaum, 2007; Juliano, 2002). The intrinsic exchange of GDP for GTP within the Rho family is relatively slow and is accelerated by their association with guanine nucleotide exchange factors (GEFs). Small Rho GTPases are known to regulate many cytoskeleton-dependent processes such as changes in cell shape, cell adhesion, cell spreading, cell migration and cell polarity (Hall, 2005). RhoA maintains the development of contractile actomyosin bundles (stress fibres) and focal adhesions, Rac1 organizes the creation of actin-rich protrusions (lamellipodia) as well as membrane ruffling, and Cdc42 regulates filopodia and the formation of peripheral actin microspikes (Hall, 2005).

Rac and Cdc42 have been implicated in the formation of invadopodia in human melanoma cells by way of a dominant active mutant of Cdc42 which enhanced dot-like degradation; whereas a dominant active mutant of Rac enhanced diffuse-type degradation (Nakahara et al., 2003). Furthermore, over-expression of frabin, a GDP/GTP exchange protein (GEF) for Cdc42 with F-actin-binding activity, enhanced both dot-like and diffuse-type degradation. However, a dominant active mutant of Rho did not affect the matrix degradation. Moreover, inhibition of phosphatidylinositol-3 kinase (PI3K) disrupted the Rac and Cdc42-dependent actin structures and blocked the matrix degradation (Nakahara et al., 2003). Additionally, the expression of activated versions of Cdc42 and Rac1 has been suggested to induce primary rat aorta vascular smooth muscle cells (VSMCs) to form extracellular matrix-degrading actin-rich protrusions that are morphologically similar to the invadopodia formed by highly invasive tumor cells (Furmaniak-Kazmierczak et al., 2007). Generation of membrane curvature is also crucial for invadopodia assembly. Recently, the F-BAR-containing protein CIP4 has been implicated in invadopodia (Pichot et al., 2010). CIP4 also binds Cdc42 and N-WASP, therefore acting as a

membrane-curving scaffolding protein. RhoA has also been implicated in invadopodia formation, however its contribution remains poorly understood; apart from a possible role in regulating exocyst binding to IQGAP1, which can also be mediated by Cdc42 (Buccione et al., 2009).

Over 70 effector proteins have been outlined to bind the Rho GTPases (Bishop and Hall, 2000; Bustelo et al., 2007). The best characterised effector proteins that bind to activated Rac and Cdc42 are the p21-activated kinases (PAKs), which were the first Rho family GTPase-regulated kinases to be identified (Manser et al., 1994). Herein, this study will focus on PAK1/2 family proteins in mediating cytoskeletal signalling events that contribute to cancer cell invasion particularly invadopodia formation.

1.6 PAK family proteins – major effectors of Rho GTPases

PAK kinases were first identified in a screen for Rac and Cdc42 effectors and independently as a proteinase-activated kinase (Manser et al., 1994; Tahara and Traugh, 1981). They are widely conserved and found in yeast as well as Drosophila (Bokoch, 2003). In mammalian cells, six isoforms of PAK kinases have been found (PAK1-6), and these are sub-divided into two groups based on biochemical and structural features **(figure 1.5).** p21-activated kinase 1 (PAK1) was the first PAK family member to be identified (Manser et al., 1994) as a serine threonine protein kinase activated by the small GTPases Cdc42 and Rac, followed by the closely related protein kinases, PAK2 and PAK3 (Bagrodia et al., 1995). More recently three other family members were discovered (PAK4-6) and the six proteins are now sub-divided into two groups **(figure 1.5)** based upon sequence and structural homology (Jaffer and Chernoff, 2002; Whale et al., 2011). PAKs are highly conserved in evolution and have many known substrates whose phosphorylation affects numerous cellular processes, including cytoskeletal organization, cell cycle progression, and cell survival (Bokoch, 2003; Dummler et al., 2009) as well as significant non-kinase related effects (Arias-Romero and Chernoff, 2008; Dummler et al., 2009).

Deletion of the *PAK1* gene in mice has no adverse effects on the viability of the foetuses but there are subtle defects in neuronal function, mast-cell degranulation and macrophage function. Genetic deletion of *PAK2* results in embryonic lethality at

day E8 due to multiple developmental abnormalities (Arias-Romero and Chernoff, 2008).

1.6.1 PAK1 and PAK2 in cancer

The *PAK1* gene is localised within chromosome region 11q13 , and 11q13.5-14 amplifications involving the PAK1 locus that have recently been reported in bladder, ovary and breast cancer (Bekri et al., 1997; Brown et al., 2008). PAK1 kinase activity is required for the Ras-induced transformation (Tang et al., 1997) and PAK1 over-expression has been reported in colon, ovarian, bladder transitional carcinoma, T-cell lymphoma and glioblastomas (Kumar et al., 2006). Indeed, glioblastoma patient survival time is significantly correlated with the presence of phosphorylated (active) PAK1 in the cell cytoplasm (Aoki et al., 2007). Important to the study herein is that PAK1 expression is widely up-regulated in human breast tumours and correlates with breast cancer invasiveness as well as tumour cyclin D1 expression (Balasenthil et al., 2004). Furthermore, PAK1 activity has been linked to estrogen (tamoxifen) resistance in estrogen receptor-positive breast cancers (Holm et al., 2006; Rayala and Kumar, 2007). These effects appear to involve the phosphorylation of the estrogen receptor on Ser305 by PAK1, and correlate with PAK1 nuclear translocation. Moreover, inducible expression of a constitutively active form of PAK1 rapidly induces breast cancer cell proliferation and aggressive cell phenotypes, which included anchorage-independent growth and mitotic defects (Vadlamudi et al., 2000). PAK1 has also been shown to have a central role in the Schwann-cell tumours of neurofibromatosis type 1 (NF1), which is caused by the loss of a Ras GAP protein, through a Ras-dependent pathway (Tang et al., 1998). Moreover, both PAK1 and PAK2 have been associated with neurofibromatosis type 2 (NF2), as PAKs phosphorylate the *NF2* tumour-suppressor gene product, Merlin, on serine 518 and block its activity (Kissil et al., 2002; Xiao et al., 2002).

1.6.2 Expression and localization of PAK1 and PAK2

Individual PAK isoforms show differences in tissue distribution and subcellular localization, which may in part account for individual substrate specificities. PAK1 is highly expressed in brain, muscle, spleen and basal expression has been reported in several tissues, including the mammary gland (Manser et al., 1994). All three

group I PAKs are highly expressed in the brain, and PAK1 and PAK2 are both highly expressed in most cells of haematopoietic origin. PDGF, insulin and certain other cell stimuli cause relocalization of PAK1 from the cytosol into cortical actin structures, such as lamellae at the leading edge, circular and peripheral dorsal ruffles in fibroblasts (Dharmawardhane et al., 1997; Sells et al., 2000), whereas PAK2 localizes to the endoplasmic reticulum (ER) in COS-7 and 293T cells (Huang et al., 2003). PAK1 localizes to the leading edge of motile neutrophils (Dharmawardhane et al., 1999), to pinocytic/phagocytic vesicles (Dharmawardhane et al., 1999; Dharmawardhane et al., 1997) and to the mitotic spindle and centrosomes (Banerjee et al., 2002; Maroto et al., 2008), as well as to the nucleus and nuclear membrane (Rayala and Kumar, 2007). In addition, PAK1 localizes to cell:substratum focal adhesions and the expression of a constitutively active form of PAK1 induces the rapid formation of lamellipodia, filopodia and dorsal ruffles, as well as increasing focal adhesion yield, and disassembly of stress fibres (Manser et al., 1997). PAK2 is expressed ubiquitously and is uniquely cleaved by caspases. The catalytic fragment generated from such cleavage translocates to the nucleus or to the endoplasmic reticulum, where it is essential for the induction of growth arrest (Huang et al., 2003).

1.6.3 PAK domain structure and function

The group I PAKs share a number of important structural features **(figure 1.5)**. All PAKs are characterised by a distinctive N-terminal regulatory domain and a highly conserved C-terminal kinase domain. The kinase domains of group I PAKs are at least 93% homologous and about 54% homologous to members of the other group. The regulatory domains of all PAKs consist of a GTPase-binding domain (GBD) and several proline-rich regions that potentially serve as docking sites for SH3 domain containing proteins. Furthermore, group I PAKs possess an autoinhibitory domain (AID) overlapping with the GBD (Zhao et al., 1998). Group I PAKs appear to form homodimers in cells, adopting a trans-inhibited conformation where the N-terminal AID of one molecule binds and inhibits the catalytic domain of the other and is important in the regulation of basal kinase activity (Lei et al., 2000). PAK3 has two alternatively spliced exons in the GBD/AID region that yield four splice variants, three of which have constitutive kinase activity (Kreis et al., 2008). These splice

variants have not been identified in PAK1/2. Active Cdc42 and Rac bind to the GBD releasing autoinhibition and enhancing kinase activity (Arias-Romero and Chernoff, 2008; Bokoch, 2003).

Signals from several growth factor tyrosine kinases including insulin, heregulin, PDGF, EGF, VEGF, HGF and G protein-coupled receptors promote activation of PAKs (Adam et al., 1998; Bagheri-Yarmand et al., 2000; Dadke et al., 2003; He et al., 2001; Royal et al., 2000; Tsakiridis et al., 1996). These pathways normally activate PAKs through sequential activation of PI-3 kinase (PI3K) and a GEF from the Dbl family, which then activates the small GTPases Rac and Cdc42. Receptor recruitment can be mediated through binding to Grb2 (Puto et al., 2003) and localization of PAK1 at the membrane is a critical step during PAK activation. However regulation of PAK1 activity is a complex process involving protein-protein interactions, phosphorylation/dephosphorylation and sphingolipid binding (Bokoch et al., 1998; Chong et al., 2001). The binding of Rac/Cdc42 to the PAK1 regulatory domain induces the phosphorylation of important sites throughout the protein, both by PAK1 itself (Chong et al., 2001) and/or by exogenous kinases such as JAK2 (Rider et al., 2007), PDK1 (King et al., 2000) and PKA (Howe and Juliano, 2000).

Indeed, phosphorylation of PAK1 serine 144 in the kinase autoinhibitory domain contributes significantly to kinase domain activation (Chong et al., 2001). Binding of Rac/Cdc42 also activates PAK2 and it is likely that the same mechanism that regulates PAK1 also regulates PAK2 catalytic activity. Like PAK1, Rac/Cdc42 interaction stimulates PAK2 autophosphorylation on Thr402 in the activation loop, a requirement for kinase activity (Gatti et al., 1999).

The adapter protein Nck (Bokoch et al., 1996) and PAK-interacting exchange factor (PIX) (Bagrodia et al., 1998) are key regulators of the group I PAKs, binding directly to PAK1-3 near the N-terminal GBD domain. Initially it was thought that Nck recruitment alone was sufficient to induce PAK kinase activity (Lu et al., 1997), though it later emerged that activation of PAK by membrane clustered Nck is dependent on Rho family GTPases (Lu and Mayer, 1999). Thus Nck serves to recruit

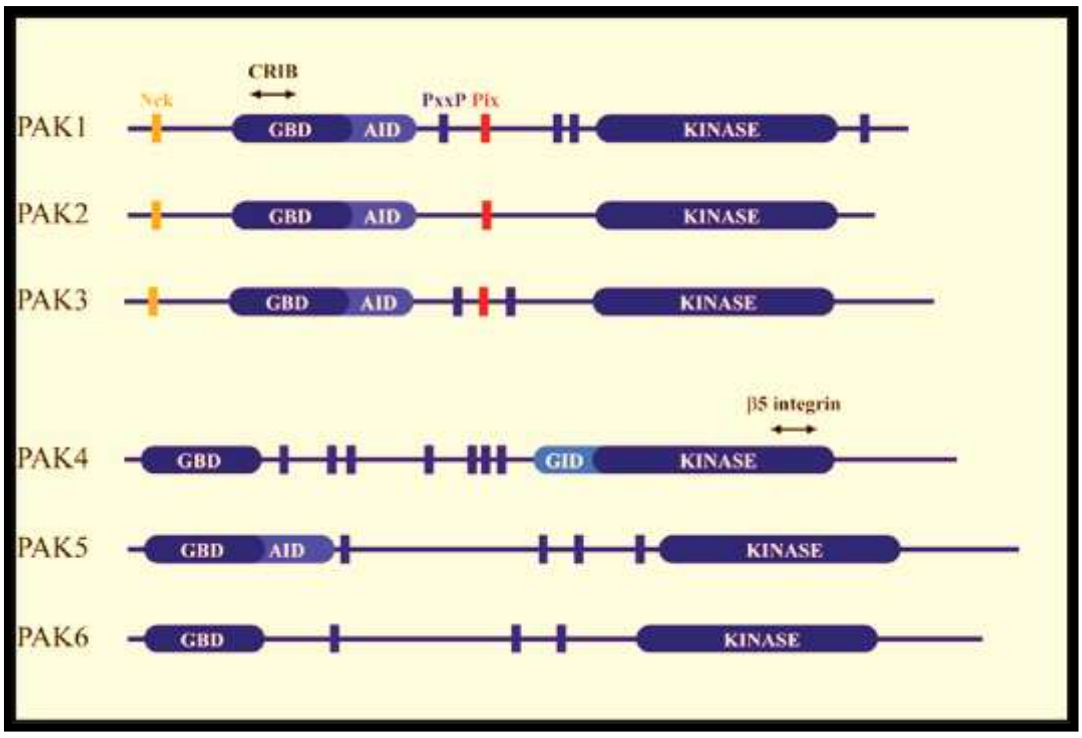

Figure 1.5 Domain structures of p21-activated kinases. All PAK family members share a common domain structure: an N-Terminal p21/GTPase binding domain (GBD) and a C-Terminal serine/threonine kinase domain. The GBD of Group I PAKs consists of a Cdc42/Rac interactive binding region (CRIB) which overlaps with an autoinhibitory domain (AID). PAK5 is the only member of the Group II PAKs that appear to contain an AID. All PAK proteins harbour variable numbers of core PxxP motifs, putative ligands for SH3 domains, although specific interacting partners are mostly unidentified. The N-Termini of the Group I PAKs bind directly to the SH3 domains of Nck1/2 via a consensus binding motif (PxxPxRxxS) indicated in orange. The Group I PAKs also harbour a PIX binding site indicated in red. Neither motif is present in any of the Group II PAKs. In addition PAK4 contains a unique GEF-H1 and Gab-1 interaction domain (GID) adjacent to the kinase domain. This kinase domain also contains a β5 integrin binding region.

PAK to areas of the cell where active Rac/Cdc42 are likely to be localised. Nck is also able to recruit a PAK1: PIX complex to sites of cell adhesion (Turner et al., 1999). PIX is a GEF for Rac/Cdc42 (Rosenberger and Kutsche, 2006) which can complex with paxillin (a major component of cell:substratum adhesions) and the interaction between PIX and PAK1 is thought to mediate adhesion dynamics by localising both active Rac/Cdc42 and PAK1 at sites of cell adhesion (Brown et al., 2002) **(figure 1.6).** Autophosphorylation of PAK1, an early event in PAK1 activation, drives the dissociation of PIX and Nck (Zhao et al., 2000) suggesting that there is a complex feedback mechanism; moreover the interaction between Nck and PAK can also be disrupted by phosphorylation of PAK on serine 21 by kinases such

as Akt (Zhou et al., 2003). In neuronal cells, PAK2 interacts with β-PIX (Hoelz et al., 2006) leading to the formation of a PAK2-β-PIX-Erk1/2 complex, which is essential for neurite outgrowth (Shin et al., 2002). Intriguingly, in this instance PAK2 inhibition blocks Rac activation, suggesting that PAK2 may also function upstream of Rac by regulating β-PIX activity (Shin et al., 2004). PAK2 is unique among the PAK isoforms because it can also be activated through proteolytic cleavage by caspases or caspase-like proteases to release an amino (N)-terminal fragment (PAK2p27) and a pro-apoptotic catalytic fragment (PAK2p34). Activation of full length PAK2 stimulates cell survival, whereas proteolytic activation of PAK2p34 is involved in programmed cell death. PAK2p34 exerts its pro-apoptotic effects via the activation of Jun N-terminal kinase (JNK) (Chan et al., 2007; Huang et al., 2009).

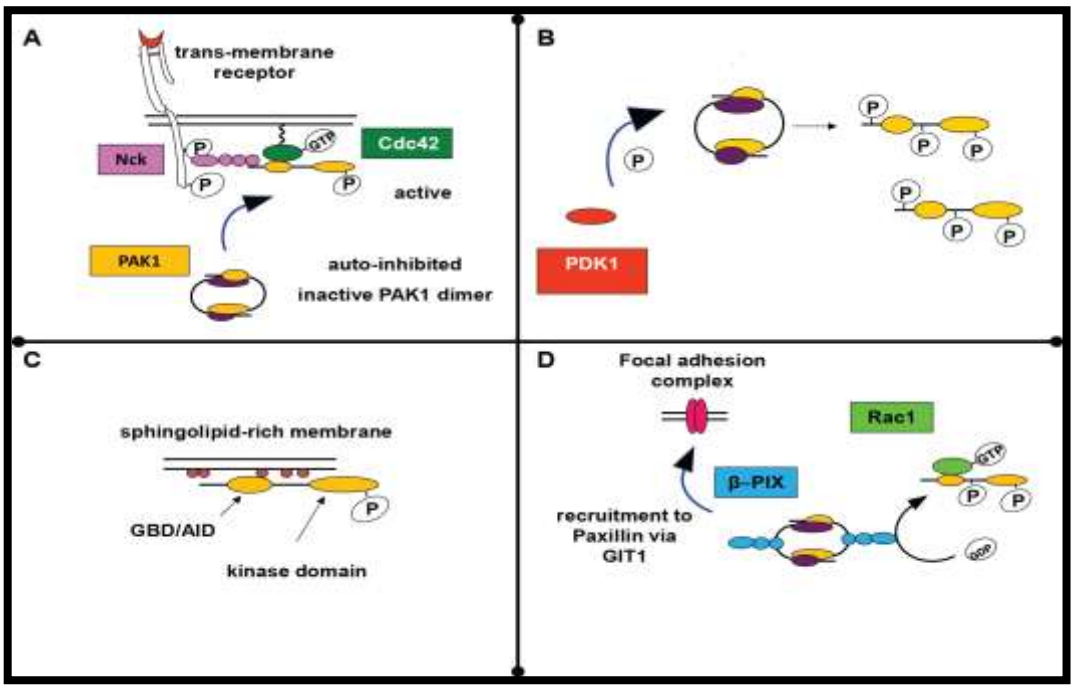

Figure 1.6 Models for activation of Group I PAKs. Group I PAKs, exemplified by PAK1, exist as autoinhibited homodimers, in which the kinase domain of one PAK molecule is inhibited by interactions from the GBD/AID domain of a second PAK molecule. (A) Upon binding to Nck bound to activated transmembrane receptor (C) spingolipids or Rho family GTPases Cdc42 or Rac (A and D) autoinhibitory interactions are relieved enabling the kinase domain to undergo conformational change and autophosphorylation to become active. PAK1, 2 and 3 are also activated by phosphorylation by additional kinases, for example PDK1 (B), which inhibit AID-kinase and PIX interaction. Note that, despite their depiction, these mechanisms may not be mutually exclusive.

1.6.4 PAK signalling to the cytoskeleton

Although the substrate preferences among group I PAKs has never been directly or systematically compared, PAK1/2/3 share 92-95% identity within their kinase domains **(figure 1.5),** suggesting that they may phosphorylate common substrates (Arias-Romero and Chernoff, 2008). Indeed, PAK1 and PAK2 have been reported to have many identical substrate *in vitro* (Rennefahrt et al., 2007). It is therefore likely that isoform-specific functions of the group I PAKs are mediated by their participation in distinct molecular complexes and their localization to distinct subcellular structures. To date, more than 30 proteins have been identified as direct substrates for group I PAKs, reflecting the important roles played by the PAK family kinases play in a variety of biological activities **(Table 1.1)**.

1.6.5 PAKs and cancer cell invasion

Group I PAKs have been implicated in cell migration through their ability to phosphorylate multiple cytoskeletal regulators. In fibroblasts, PAK1 regulates lamellipodial extension and directionality (Sells et al., 1999; Sells et al., 2000) and the formation and disassembly of focal adhesions (Manser et al., 1997; Nayal et al., 2006). In contrast, in endothelial cells both kinase dead and constitutively active PAK1 inhibited migration (Kiosses et al., 1999), indicating that the role of PAKs in cell migration is likely to be cell-type specific. In prostate cancer cells, knockdown of PAK1 inhibits HGF-induced loss of cell:cell junctions and subsequent migration whilst knockdown of PAK2 increases lamellipodium extension but does not affect migration speed (Bright et al., 2009). However, expression of either kinase-dead or constitutively active PAK1 has been shown to increase migration towards HGF in Boyden chambers (Zegers et al., 2003). siRNA-mediated knockdown of PAK1 in breast epithelial cells leads to decreased myosin light chain phosphorylation and smaller focal adhesions whilst dominant-negative PAK1 blocks the invasiveness of breast tumour cells (Adam et al., 2000). In contrast, knockdown of PAK2 has the opposite effects (Coniglio et al., 2008) suggesting they could have antagonistic roles. Interestingly, PAK1 has also been shown to co-ordinate extracellular matrix proteolysis in a 3D breast cancer model (Li et al., 2008). Moreover, a recent study

Substrate	Cellular function	PAK	reference
Caldesmon	Inhibitor of myosin ATPase activity	PAK1 & 3	(Foster et al., 2000)
CPI17	Inhibitor of myosin phosphatase	PAK1	(Takizawa et al., 2002)
Desmin	Intermediate filament protein	PAK1	(Ohtakara et al., 2000)
Filamin A	Actin cross linking and adhesion protein	PAK1	(Vadlamudi et al., 2002)
GIT1	GTPase regulation Arf GAP	PAK1	(Zhao et al., 2005)
GEF-H1	Rho GTPase regulation, RhoA GEF	PAK1	(Zenke et al., 2004)
LIMK1	Actin cytoskeleton dynamics; cofilin kinase	PAK1 & 2	(Edwards et al., 1999)
MLCK	Regulation of myosin activity and actin cytoskeleton dynamics	PAK1 & 2	(Goeckeler et al., 2000; Sanders et al., 1999)
Merlin	ERM binding protein	PAK2	(Kissil et al., 2002)
p41-ARC	Subunit of Arp2/3 complex, actin nucleation	PAK1	(Vadlamudi et al., 2004)
Paxillin	Focal adhesion scaffold	PAK1 & 3	(Turner et al., 1999)
α-PIX	Rho GTPase regulation, Rac GEF	PAK1 & 2	(Chong et al., 2001)
β-PIX	Rho GTPase regulation, Rac GEF	PAK1 & 2	(Shin et al., 2002; ten Klooster et al., 2006)
Raf-1	MEK kinase	PAK1 & 3	(King et al., 1998; Zang et al., 2002)
Rho-GDI	Inhibitor of Rho GTPase activity	PAK1	(DerMardirossian et al., 2004)
R-MLC	Regulatory chain of myosin motor	PAK2	(Chew et al., 1998)
Vimentin	Intermediate filament protein	PAK1	(Goto et al., 2002)
Cortactin	Regulation of branched-actin filaments dynamics	PAK1	(Vidal et al., 2002)

Table 1.1. PAK kinase substrates implicated in invasive migration

reported that PAK1 and PAK2 are involved in promoting cell migration and invasion in ovarian cancer cells (Siu et al., 2009). Cancer cell dissemination may require a loss of cell: cell contact and PAK1 kinase mutants can induce a loss of cell: cell junctions (Zegers et al., 2003). Additionally, active Rac acts via PAK1 to induce disassembly of E-cadherin-based adhesions (Lozano et al., 2008), a process that may depend on an interaction between PAK1 and E-cadherin associated protein β-catenin (He et al., 2008).

1.6.6 PAKs and invadopodia

PAK1 acitivity was found to be required to sustain invadopodia formation and activity in invasive human melanoma cells (Ayala et al., 2008) . The autoinhibitory domain of PAK1 (PAK1-AID), known to inhibit the endogenous kinase (Zhao et al., 1998) was found to induce a substantial decrease in ECM degradation *in vitro*, where cortactin has been shown to be a substrate for PAK1 (Vidal et al., 2002) and the main residue targeted by PAK1 is serine 113 (Webb et al., 2006). Co-expression of PAK-AID with pseudophosphorylated cortactin^{S113D} was not sufficient to bypass the block induced by PAK-AID (Ayala et al., 2008). This suggests that PAK1 activity supports invadopodia formation.

Invadopodia formation in VSMCs could also be potently induced by kinase-dead PAK1 (Furmaniak-Kazmierczak et al., 2007). In these same cells, over-expression of PAK1 kinase mutants induces podosome formation, and interaction with β-PIX is implicated to be necessary for localization of PAK1 to the podosomal ring structure (Webb et al., 2005). Similarly, cellular PAK4 localizes to macrophage podosomes (Gringel et al., 2006). Src and ERK kinase activity were also necessary for kinase dead PAK1 to promote invadopodia formation. The requirement of ERK is interesting because PAK1 directly binds ERK and facilitates its activation in VSMCs (Sundberg-Smith et al., 2005). Constitutively active PAK1 only weakly promotes invadopodia formation suggesting that its ability to phosphorylate substrates such as MLCK, LIMK, paxillin and vimentin may actively repress invadopodia formation (Furmaniak-Kazmierczak et al., 2007). However, to date, there is no evidence to show a direct effect of altered PAK1/2 expression levels on invadopodia formation and matrix degradation in breast carcinoma.

1.7 Aims of the project

1. To use carcinoma cell lines and a 2D in vitro matrix degradation assay to determine

a) whether invadopodia formation can be induced by growth factor stimulation
b) whether invadopodia formation can be induced by increasing intracellular levels of HIF-1α (hypoxia)
c) whether Group I PAKs regulate invadopodia formation downstream of growth factor and/or hypoxia signalling

2. To use a PCR array to investigate cytoskeletal gene changes following increased intracellular HIF-1α levels.

Chapter 2 – Materials and Methods

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 General reagents

Reagent	Company
Agarose	Sigma-Aldrich,UK
Alexa Fluor®488 phalloidin	Invitrogen,UK
Alexa Fluor®633 phalloidin	Invitrogen,UK
Ampicillin	Sigma-Aldrich,UK
Bromophenol blue	Sigma-Aldrich,UK
Calcium Phosphate Transfection Reagent	Invitrogen,UK
Cell-dissociation solution (1x) non-enzymatic	Sigma-Aldrich,UK
DH5α competent cells	Invitrogen,UK
DMEM (Dulbecco's Modified Eagle's Medium)	Sigma-Aldrich,UK
DMEM +Glutamax™-1	Gibco,UK
DMEM F-12 (Medium Ham's F-12)	Lonza,UK
DMOG	Frontier Scientific,UK
DMSO	Sigma-Aldrich,UK
DPBS (Dulbecco's Phosphate Buffered Saline 1x)	GIBCO, UK
DTT	Sigma-Aldrich,UK
ECL Nitrocellulose membrane	Perkin Elmer, USA
ECL Western Blotting Detection reagent	Pierce,UK
EGF (Recombinant Human)	R&D systems,USA
Gateway™ LR and BP Clonase™ Enzyme Mix	Invitrogen,UK
Gelatin- Type A from porcine skin	Sigma-Aldrich,UK
Gentamycin	Sigma-Aldrich,UK
Glass coverslips 13mm	VWR International,UK
Glutaraldehyde solution, Grade I, 25%	Sigma-Aldrich,UK
Glycerol	Sigma-Aldrich,UK
Glycine	Sigma-Aldrich,UK

HGF (Recombinant human)	R&D systems,USA
Hyperfilm™ ECL™ X-ray film	Fujifilm
Invitrogen Mini and Maxi-prep Kits	Invitrogen,UK
Lipofectamine 2000 transfection reagent	Invitrogen,UK
LB agar and LB broth	Sigma-Aldrich,UK
Menzel-Glaser Glass Slides	Thermo scientific,UK
Parafilm	Pechiney Plastic Packaging,UK
Paraformaldehyde	Sigma-Aldrich,UK
Phosphate buffered saline (PBS) (DPBS)	Invitrogen,UK
Phosphate buffered saline (PBS) tablets	Oxoid Limited,UK
QIAquick Gel Extraction Kit	Qiagen Ltd,UK
Quickchange Site-directed Mutagenesis kit	Stratagene
Rhodamine B Isothiocyanate	Sigma-Aldrich,UK
RPMI-1640	GIBCO, UK
S.O.C media	Invitrogen,UK
Slide-A-Lyzer® Dialysis Cassette, 12-30ml	Pierce Biotechnology,UK
Sodium borohydride	Sigma-Aldrich,UK
Sodium Chloride	Sigma-Aldrich,UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich,UK
Stripping buffer	Sigma-Aldrich,UK
Sucrose	Sigma-Aldrich,UK
Tris base	Sigma-Aldrich,UK
TRITC-Phalloidin	Sigma-Aldrich,UK
Triton X-100	Sigma-Aldrich,UK
Trypsin-EDTA 0.5% (1x) (GIBCO)	Invitrogen,UK
Tween 20	Sigma-Aldrich,UK

Table 2.1 General reagents and kits

2.1.2 Mammalian cell lines

A375MM (metastatic melanoma cells) was obtained from Dr. Ester Martin, King's College London, MCF-7 and MDA-MB-231 (breast adenocarcinoma cells), HT29 (colon carcinoma cells), PC3 and DU145 (prostate carcinoma cells) and HEK-293 (human embryonic kidney cells) were obtained from ATCC.

2.1.3 Plasmids

Mouse-Myc-PAK1 was obtained from Prof. Anne Ridley

Human-Myc-PAK1/PAK2 were obtained from Dr. Jonathan Chernoff

GFP-PAK1, GFP-PAK2, GFP-HIF-1α expression plasmids and HA-PAK2^{r} mutant were generated using Gateway™ Technology

pDEST™GFP and HA

pDONOR™207

Primers were ordered from Thermo Fisher Scientific,Germany

2.1.4 Antibodies

Antibody	Company	Source	Application	Dilution
Anti-c-Met	Santa Cruz	Rabbit	Western Blotting	1:500
Anti-EGFR	Cell Signaling	Rabbit	Western Blotting	1:500
Anti-PAK1	Cell Signaling	Rabbit	Western Blotting	1:2000
Anti-PAK2	Cell Signaling	Rabbit	Western Blotting	1:2000
Anti-Cortactin	Upstate	Mouse	Immunofluorescence	1:50
Anti-ERK	Cell Signaling	Rabbit	Western Blotting	1:2000
Anti-Myc	Santa Cruz	Mouse	Immunofluorescence	1:50
Anti-HA	Santa Cruz	Mouse	Immunofluorescence	1:50
Anti-phospho-PAK1/2	Cell Signalling	Rabbit	Western Blotting	1:1000
Anti-Gapdh	Santa Cruz	Mouse	Western Blotting	1:10000
Anti-HIF-1α	Cell Signalling	Rabbit	Western Blotting	1:1000
Anti-HIF-2α	Santa Cruz	Mouse	Western Blotting	1:500
Anti-Cool1/β-PIX	Cell Signalling	Rabbit	Western Blotting	1:1000
Anti-E-cadherin	Gene Tax	Mouse	Immunofluorescence	1:50
Anti-E-cadherin	Gene Tax	Mouse	Western Blotting	1:2000

Table 2.2 Primary antibodies

Antibody	Company	Source	Application	Dilution
HRP conjugated anti mouse	Dako	Goat	Western Blotting	1:2000
HRP conjugated anti rabbit	Dako	Goat	Western Blotting	1:2000
Alexa Fluor® 488 anti-rabbit IgG	Invitrogen	Goat	Immunofluorescence	1:200
Alexa Fluor® 488 anti-mouse IgG	Invitrogen	Goat	Immunofluorescence	1:200
Alexa Fluor® 568 anti-rabbit IgG	Invitrogen	Goat	Immunofluorescence	1:200
Alexa Fluor® 568 anti-mouse IgG	Invitrogen	Goat	Immunofluorescence	1:200

Table 2.3 Secondary antibodies

Solution	Composition
Blocking solution	5% w/v milk powder or 5% w/v bovine serum albumin in TBS-Tween
DNA loading buffer	40% w/v sucrose, 0.25% bromophenol blue
SDS-PAGE running buffer (10X)	Tris Base, glycine, SDS Dilute to 1X with dH_2O
SDS-PAGE sample buffer	100mM Tris pH 6.8, 10% w/v SDS, 30% v/v glycerol, 0.2% w/v bromophenol blue, 2% v/v β-mercaptoethanol
SDS-PAGE transfer buffer (10X)	0.39M glycine, 0.48M Tris base, 12.8mM SDS Make up to 1X transfer fresh on the day by diluting to 1X and adding methanol to a final concentration of 20% v/v
TAE buffer	40mM Tris acetate, 10mM EDTA
NP-40 Lysis Buffer	1M Tris pH7.6, 5M NaCI, 0.5M EDTA, 10% NP-40 and add protease inhibitor cocktail fresh each time before use
Protease inhibitor cocktail	1mM sodium orthovanadate, 50mM sodium fluoride, 1mM PMSF, 1µg/ml Aprotinin, 20µg/ml leupeptin, 1mM DTT
Stripping buffer	25mM glycine pH2.0, 1% SDS
PBS-Tween	PBS + 0.1% Tween-20
TBS-Tween	50mM NaCI, 0.1% v/v Tween, 25mM TRIS pH7.6

Table 2.4 General buffers and solution

2.2 Methods

2.2.1 Mammalian Cell culture and maintenance

DU145 and PC3 cells were cultured in 10% FBS, 90% RPMI-1640, penicillin-streptomycin, L-Glutamine. MDA-MB-231, HT29, MCF-7 and HEK-293 cells were cultured in 10% FBS, 90% DMEM + Glutamax™-1 and penicillin-streptomycin. A375MM cells were cultured in 10% FBS, 90% DMEM F-12, 15mM Hepes, penicillin-streptomycin and L-glutamine. These cells were incubated at 37ºC in a tissue culture incubator with humidified air, supplemented with CO_2 to 5% over atmospheric levels. Adherent cells were grown in a $75cm^2$ vented tissue culture flasks until 80% confluent. The complete growth media was removed and the cells were washed with 10ml sterile PBS before being incubated with 3 ml trypsin/EDTA at 37ºC for about 2 minutes until they detached and checked by microscopy. 10 ml of complete media was then added to the cells to inactivate the trypsin. Cells were pelleted by centrifugation at 1000rpm for 5 minutes, the media removed and the cell pellet resuspended in 5ml complete media. Dilutions of the suspended cells were placed in a fresh tissue culture flask and the volume made up to 20ml in complete media. All media were warmed to 37ºC before use.

2.2.2 Freezing and thawing cells

Cells were harvested and re-suspended in 45% FBS and 45% complete growth medium with 10% DMSO. Cells were frozen overnight at -80ºC in a cryo freezing container before they were placed in a liquid nitrogen tank. When taken out of the nitrogen tank, cells were thawed rapidly at 37ºC. Cells were added to pre-warmed medium, then were gently centrifuged at 1000 rpm for 4 minutes to remove the DMSO and finally re-suspended in fresh complete growth medium for culturing.

2.2.3 Cell treatments

For growth factor stimulations, MCF-7 and MDA-MB-231 cells were seeded onto 6-well plates at a density of $3X10^4$ cell/ml in 2mls media and incubated overnight to adhere. Cells were then incubated for 24 hours in 0.5% FBS media prior to being re-seeded on gelatin-coated coverslips with 3 hours EGF (100ng/ml) or HGF (10ng/ml) stimulation. For PAK activation downstream of EGF/HGF, cells were stimulated for up to 1 hour followed by cell lysis. For HIF-1α induction, dimethyloxaloylglycine

(DMOG), a prolyl-4-hydroxylase inhibitor shown to upregulate the HIF activity (Asikainen et al., 2005) was used. 500mM stock of DMOG was prepared in dH_2O and stored in -20ºC. A working concentration of 0.5mM DMOG was used to treat the cells on plastic dishes for up to 6 hours. Following treatment, cells were either lysed or re-seeded on gelatin-coated coverslips.

2.2.4 Cell Lysis

Treated or non-treated cells were lysed in NP-40 lysis buffer on ice for 10 minutes and centrifuged at 13000 rpm for 10 minutes to remove cell debris. Cell lysates were mixed with 2X SDS sample buffer, boiled at 95ºC for 3 minutes and used either immediately or stored at -20ºC. Proteins were resolved by SDS-PAGE and immunoblotted for the desired protein.

2.2.5 Immunoblotting

Up to 30µl of whole cell lysates were loaded in each well on SDS-PAGE gels covered with 1x running buffer and migrated through the gel using 125V. After electrophoresis proteins were transferred from the gel onto a nitrocellulose membrane in 1X SDS-PAGE blotting buffer using a wet transfer system (Biorad). Proteins were transferred at 100V for 1hour in cold transfer buffer. For immunodetection, membranes were then blocked in 5% milk/TBS-Tween for one hour at room temperature with rocking to prevent non-specific antibody binding. Then the membrane was washed in TBS-T and incubated with primary antibodies overnight at 4ºC on the roller (for antibody dilutions see Table 2.2). Following three washes in TBS-Tween for 10 minutes, the blots were incubated with the appropriate horseradish peroxidise-conjugated (HRP) (Dako) secondary antibodies for one hour at room temperature (for antibody dilutions see Table 2.3). After three more washes with TBS-T for 10 minutes, immunoprobed proteins were visualized by Enhanced Chemiluminescent Reagents (ECL Pierce) and exposed to Fuji X-Ray films followed by developing using Imaging systems Xograph compact X4 developer. To re-probe the membrane with another primary antibody, membranes were incubated in stripping buffer twice for 15 minutes followed by 5 minutes with PBS-Tween at room temperature, then blocked again in 5% milk or BSA in TBS-T for one hour and incubated overnight with new primary antibodies at 4ºC.

2.2.6 Quantification of blots

To quantify the changes in phosphorylation levels, protein bands exposed onto ECL films were scanned onto computers and save as TIF files. The intensity of each phospho-protein and total protein band was determined using Andor IQ software from which the phospho-protein: total protein ratio was calculated and normalised to that of the starved only cells. Mean and SEM values were calculated from the data of 3 independent experiments.

2.2.7 Treatment of glass coverslips for invadopodia assay

13mm diameter coverslips were gently placed in 70% ethanol for 30 minutes with rocking, and both surfaces of the coverslips were fully covered with the ethanol preferably in a 10cm dish. The 70% ethanol was then poured off followed by adding 96% ethanol onto the coverslips for 30 minutes. In tissue culture hood, the coverslips were left to dry on paper towel before being placed in a fresh 10cm dish. The coverslips were kept sterile in the dish at room temperature for long term storage.

2.2.8 Preparation of fluorophore-conjugated gelatin

Fluorophore-conjugated gelatin is used to coat coverslips prior to seeding cells onto them for the invadopodia assay. This preparation is performed according to (Mueller and Chen, 1991), with the following modifications. 100mg gelatin is dissolved in a total of 50ml buffer mix containing 61mM sodium chloride (NaCI) and 50mM sodium borohydride ($Na_2B_4O_7$) (pH 9.3) and then incubated at 37ºC for one hour. After incubation time, 1.8mg/ml of rhodamine ITC (or FITC) is added and mixed for 4 hours in complete darkness at room temperature on the roller. This mixture is then dialysed in PBS overnight at 4ºC in complete darkness. Dialysis is usually repeated for 2 days with 3 buffer changes per day. After a quick spin to remove insoluble material and addition of 2% w/v sucrose, small aliquots are stored in the dark at 4ºC.

2.2.9 Preparation of fluorophore-conjugated gelatin coated coverslips

Fluorophore–conjugated gelatin coated coverslips are prepared and the assay carried out as described (Baldassarre et al., 2003; Bowden et al., 2001; Mueller and Chen, 1991). Fresh gelatin-coated coverslips were prepared each time before seeding cells onto them. Sterile ethanol-washed coverslips were placed in a 24-well plate. The

coverslips were coated with pre-warmed fluorophore-conjugated gelatin solution, using enough to cover the surface, spread to the edges and incubated at room temperature in the dark for 10 minutes. 0.5% glutaraldehyde in PBS was prepared from 25% stock. A drop of 200µl of glutaraldehyde for each coverslip was dotted onto parafilm. The gelatin-coated coverslips were inverted onto the dots and incubated in the dark to crosslink for 15 minutes. Each coverslips were then transferred to a new 24-well plate with the coated side up, and gently washed for 3 times with sterile PBS. The coverslips were finally incubated with 5mg/ml sodium borohydride prepared in PBS for 3 minutes at room temperature in darkness followed by 3 times PBS washes. Washed coverslips were then sterilized again in 70% ethanol for 5 minutes. The ethanol was aspirated off and the coverslips are dried for 10 minutes under a sterile hood before quenching in DMEM medium for 1 hour at 37ºC. At this stage, coverslips are ready for seeding cells.

2.2.10 Invadopodia Assay

Media was removed from treated or un-treated cells in culture plates. Adherent cells were washed once with sterile PBS. Non-enzymatic cell dissociation buffer was added into each well and incubated at 37ºC for 10 minutes until the cells detached from the bottom of the plate. Dislodged cells were then re-suspended in culture medium. Cells were counted and the number of viable cells was noted. The media on the coverslips were aspirated off and $2x10^4$ cells/ml were seeded onto them (1ml on each coverslips). Cells were then incubated at 37ºC for 3 hours before fixing the coverslips with 4% paraformaldehyde for 20 minutes at room temperature. During this incubation, treatment with 10ng/ml HGF, 100ng/ml EGF or 0.5mM DMOG was performed accordingly. Coverslips from 3 independent experiments in all cell lines were prepared in this way before they were all identically stained for F-actin with Alexa Fluor® 488 or 633 Phalloidin. Cells were imaged on an Olympus 1X71 fluorescence microscope and LSM 510 Zeiss Confocal microscope. For invadopodia quantification, invadopodia images on the F-actin and gelatin were taken using the same fluorescence microscope settings. Cells were scored for the presence of underlying invadopodia and the mean percentage of cells with invadopodia formation calculated.

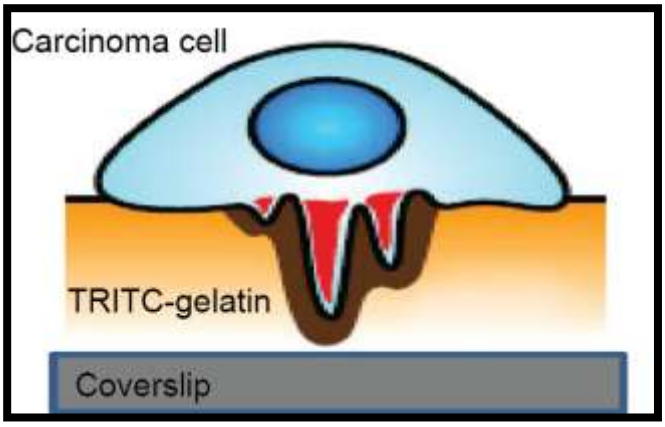

Figure 2.1: Invasive carcinoma cells formed invadopodia that extended into the matrix substratum.

2.2.11 Immunofluorescence staining

Fixed cells were gently washed 3 times with PBS. The coverslips were permeabilised in 0.2% triton X-100/PBS for 5 minutes followed by 3 more PBS washes. Alexa Fluor® 488/633 Phalloidin were diluted in PBS at (1:500). The coverslips were then incubated with diluted Phalloidin for 1 hour at room temperature in dark humidified environment. For double staining with primary and secondary antibodies, fixed, PBS washed and permeabilised coverslips were blocked with 3% BSA in PBS for 30 minutes to block for non-specific bindings followed by 3 times washes with PBS. Primary antibody was diluted with 3% BSA in PBS. The coverslips were then incubated with diluted primary antibody for 2 hours at room temperature in dark humidified environment followed by 3 times washes with PBS. Secondary antibody was also diluted in the same manner plus conjugated-phalloidin if it's required. The coverslips were incubated with diluted secondary antibody for 1 hour at room temperature in dark humidified environment. Coverslips were washed twice with PBS, then once with dH_2O to prevent salt crystals and mounted onto glass slides with anti-fade mounting solution. The set coverslips were stored at 4ºC or -20ºC for long term storage.

2.2.12 Gelatin degradation analysis

ImageJ software (gelatin degradation plug-in) was used to measure area of matrix degradation per invadopodia forming cells. Cells and gelatin images were converted to 8-bit copy prior to combining all cell and gelatin images in separate stacks (cell

images in one stack and gelatin images in another stack). These stacks were then opened in ImageJ software to measure total degradation area per cell. Data will then be multiplied by pixel area (μm^2) to get real degradation area.

2.2.13 Calcium phosphate transfection of adherent HEK-293 cells

HEK-293 cells were seeded at a density of 1 x 10^5 cells/ml in 10mls into any of the dishes shown in Table 2.5. The plate was incubated at 37ºC for 24 hours. On the following day, 3 to 4 hours prior to transfection, the media on the plate was changed with a fresh media. The transfection mixture was made following the recipe in Table 2.5.

10cm dish (10mls)	6cm dish (5mls)	2cm dish (2mls)/ 6-well plate
To a tube labelled A add	To a tube labelled A add	To a tube labelled A add
36µl 2M $CaCI_2$	18µl 2M $CaCI_2$	7.2µl 2M $CaCI_2$
20µg DNA	10µg DNA	4µg DNA
Make volume to 300µl with sterile water	Make volume to 150µl with sterile water	Make volume to 60µl with sterile water
To a tube labelled B add 300µl 2x Hepes buffered saline (HBS)	To a tube labelled B add 150µl 2x Hepes buffered saline (HBS)	To a tube labelled B add 60µl 2x Hepes buffered saline (HBS)

Table 2.5: HEK-293 calcium phosphate transfection mixture recipe

Using a pipette, A was added to B slowly drop-wise with aeration until A was depleted. The mix was incubated at room temperature for 30 minutes. The plate with cells was taken from the incubator and the transfection mix was added to the cells drop-wise and the plate was dispersed for even spreading of the transfection reagent. The transfected cells were incubated at 37ºC overnight. Media was removed and replaced with fresh media the following day and reincubated for another 24 hours. Cells were fixed with 4% paraformaldehyde followed by staining with specific antibodies.

2.2.14 RNAi transfection

MDA-MB-231 cells were seeded at $4X10^4$ cell/ml in 2mls complete growth medium, allowed to adhere for 24 hours and transfected with siRNA oligos using HiPerfect according to the manufacturer's protocol. Cells were transfected with 75nM small interfering (si) RNA targeting HIF-1α (Ambion, Catalogue number 4392420) for 48 hours and 75nM non-specific siRNA (Qiagen) as control. For β-PIX siRNA, 75nM of a pool of 4 siRNA duplexes each designed to target human β-PIX (siGENOME SMARTpool) (Dharmacon, Catalogue number 8874) was transfected for 24 hours.

2.2.15 Transfection using Lipofectamine 2000

Lipofectamine 2000 transfection reagent (Invitrogen) was used to transfect PAK1/PAK2 shRNA oligos, GFP-PAK1/PAK2, GFP-HIF-1α, mouse-myc-PAK1 and HA-$PAK2^r$ DNA constructs. Cells were plated the day before transfection to allow them to adhere overnight. Prior to transfection, complete growth media was replaced with antibiotics-free growth media. Between 6-8 hours after the transfection mix was added to cells, the medium was replaced with fresh complete growth medium. Depending on the assay, cells were either re-seeded on fluorophore-conjugated gelatin coverslips or lysed between 24-48 hours after transfection.

2.2.16 Hypoxic and normoxic conditions

MDA-MB-231 cells grown in complete growth medium were initially maintained in normoxia, humidified air (20% O_2, 5% CO_2 and balanced N_2 incubator) at 37°C. In the case of hypoxia stimulations, cells were transferred from normoxia to a Galaxy R (New Brunswick Scientific) CO_2 incubator under an atmosphere mixture (1.5% O_2, 94% N_2 and 5% CO_2) at 37°C for the specified amount of time.

2.2.17 Transformation of *E.coli* cells

DH5α and DB3.1 strains of *E.coli* cells were transformed with DNA plasmids by the process called heat shock. Competent *E.coli* cells stored at -80ºC were thawed out slowly on ice prior to the addition of 1-10 μg of plasmid DNA. Bacteria with the added DNA were incubated on ice for 30 minutes, placed at 42ºC for 30 seconds and then quickly returned to the ice. S.O.C media was added to the transformed bacteria and incubated at 37ºC with shaking for 1 h. The bacteria were then plated onto L-agar supplemented with the required antibiotic and incubated overnight at 37ºC.

Name	Target gene	Target sequence	Company
shPAK1	PAK1	GCCTAGACATTCAAGACAA	Open Biosystems
shPAK2	PAK2	CAATATTTCGGGATTTCTT	Open Biosystems
shControl (pGIPz)			Open Biosystems
siHIF-1α	HIF-1α	5'-GATAAAAGGTTACAAACGAtt-3' 5'-TCGTTTGTAACCTTTTATCtg-3'	Ambion
siGENOME SMARTpool siβ-PIX	β-PIX	GGAAGAAGAUGCUCAGAUU GAAGAGCCCUCCCAAAGGA UCAAAGAGCUCGAGAGACA GGAGGGCGAUGACAUUAAA	Dharmacon
Non-targeting siRNA		AATTCTCCGAACGTGTCACGT	Qiagen

Table 2.6 RNA interference and oligonucleotide sequences

2.2.18 Purification of plasmid DNA

Invitrogen mini- and maxi-prep kits were used to isolate and purify plasmid DNA from *E.coli* cells. Transformed bacteria single colony was inoculated into L-broth supplemented with the required antibiotic grown in either 5ml (for mini prep) or 200ml (for maxi prep) of LB broth at 37ºC overnight with shaking. Mini-prep kits were typically used to isolate 20μg of plasmid DNA from 1.5ml of L-broth while maxi-prep kits were used to isolate up to 500μg from 250ml. Plasmid DNA was eluted in TE from Invitrogen columns and stored at -20ºC.

2.2.19 PCR reaction

HIF-1α human cDNA clone was purchased from Origene,UK and used as the cDNA template in the generation of HIF-1α cDNA flanked by attB sequences. PAK1/PAK2 were cloned out from the CMV-Myc vectors in the generation of PAK1/PAK2 cDNA flanked by attB sequences. attB sequences were added to enable cloning into

Gateway™ vectors. cDNAs flanked by attB sequences were generated by PCR amplification using the following primers:

Name	Experiment	Sequence 5'-3'
HIF-1α attB1 forward	Cloning of GFP- HIF-1α	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGAGGGCGCCGGCGGCGCG
HIF-1α attB2 reverse	Cloning of GFP- HIF-1α	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTTAACTTGATCCAAGC
PAK1 attB1 forward	Cloning of GFP-PAK1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGTCAAATAACGGCCTAGAC
PAK1 attB2 reverse	Cloning of GFP-PAK1	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGCTGCAGCAATCAGTGGA
PAK2 attB1 forward	Cloning of GFP-PAK2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGTCTGATAACGGAGAACTG
PAK2 attB2 reverse	Cloning of GFP-PAK2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAACGGTTACTCTTCATTGC
PAK2^{r} attB1 forward	Site directed mutagenesis of HA-PAK2^{r}	GAGAAACTTTCCCCAATATTTCGAGATTTCTTAAATCGATGTTTGGA
PAK2^{r} attB2 reverse	Site directed mutagenesis of HA-PAK2^{r}	TCCAAACATCGATTTAAGAAATCTCGAAATATTGGGGAAAGTTTCTC
Actin forward	RT-PCR	CATGTACGTTGCTATCCAGGC
Actin reverse	RT-PCR	CTCCTTAATGTCACGCACGAT
β-PIX forward	RT-PCR	ATGAATTCCGCCGAGCAAACC
β-PIX reverse	RT-PCR	TTAGTTCTGGTGAGAGATATA
VEGF forward	RT-PCR	CTTGCCTTGCTGCTCTACCT
VEGF reverse	RT-PCR	CTGCATGGTGATGTTGGACT

Table 2.7 Primer sequences

The Polymerase chain reaction (PCR) was conducted using AccuPrime™ *Taq* DNA Polymerase (high fidelity mix containing 200mM Tris-HCI (pH 8.4), 500mM KCI, 15mM $MgCI_2$, 2mM dGTP, 2mM dATP, 2mM dTTP, 2mM dCTP, thermostable AccuPrime™ protein, 10% glycerol). 200ng template DNA and 200nM of forward and reverse primers were used. The specific conditions of the PCR reaction used are shown in Table 2.8.

Cycle/s	Process	HIF1α
1	Pre-incubation (Initial denaturation)	95ºC, 5 minutes
30	Denaturation	95ºC, 15 seconds
	Annealing	56ºC, 30 seconds
	Extension	68ºC, 2minutes 30 seconds
1	Final Extension	68ºC, 10 minutes

Table 2.8: Conditions for PCR of HIF-1α

2.2.20 Gel purification of DNA fragments

DNA samples were diluted in 10X loading buffer containing glycerol and bromophenol blue. PCR products were resolved on 1% w/v agarose gel (w/v) boiled in 1x TAE (0.04M Tris acetate pH 8, 0.01M EDTA) buffer containing 0.5µg/ml ethidium bromide, to separate reaction products mixed with 3µl of 5x loading dye. Samples were then separated at 130V in 1X TAE running buffer. The DNA was visualised under low intensity UV light (trans-illuminator). The desired fragment was excised from the gel with a razor and the DNA isolated from the agarose using a QIAquick gel extraction kit according to the manufacturer's instructions.

2.2.21 Construction of entry clone

A Gateway™ BP Recombination Reaction was set up between pDONOR™207 vector and the attB sequence flanked PCR product according to manufacturer's instructions to produce pENTRY-plasmid. The reaction was incubated at room temperature for 1 hour before being terminated by the addition of Proteinase K for 10 minutes at 37ºC. The reaction mixture was transformed into DH5α *E.Coli* cells and the plasmid DNA from individual colonies was isolated and screened by PCR.

DNA from positive clones was sent for sequencing to Eurofins MWG Operon's sequencing service, Germany using sequencing primers to verify insertion of the correct cDNA.

2.2.22 Construction of GFP- HIF-1α, PAK1/2 expression clones

To construct the expression plasmids, a Gateway™ LR Recombination Reaction was set up between the pENTRY entry clones and the pDEST destination vector (Invitrogen) according to manufacturer's instructions. The LR reactions were incubated at room temperature for 1 hour and terminated by the addition of Proteinase K. The reaction mixture was transformed into DH5α *E.Coli* cells and the plasmid DNA isolated from individual colonies and screened by PCR. DNA from positive clones was subsequently sent for sequencing to Eurofins MWG Operon's sequencing service, Germany using sequencing primers.

2.2.23 Site-directed mutagenesis

The pENTRY-PAK2 plasmid was generated using the Gateway Cloning system (Invitrogen). Point mutations were introduced into the plasmid using the site-directed mutagenesis kit from Stratagene following the manufacturer's instructions. Primers were designed including the desired mutations (listed in Table 2.7). A 50µl reaction was set up containing 25ng plasmid DNA, 125ng of each primer, 1µl dNTP mix, 5µl 10X reaction buffer and 1µl PfuUltra DNA polymerase. A PCR program with the following parameters was used:

Cycle/s	Process	PAK1/2^{r}
1	Pre-incubation (Initial denaturation)	95ºC, 30 seconds
18	Denaturation	95ºC, 30 seconds
	Annealing	65ºC, 1 minute
	Extension	68ºC, 6minutes

Table 2.9: Conditions for mutagenesis PCR

2.2.24 RT-PCR expression analysis

Total cellular RNA was isolated using an RNeasy Plus Mini Kit (Qiagen). Template cDNAs were synthesized with Superscript III (Invitrogen). Semi-quantitative reverse-transcriptase (RT-PCR) was performed with Red-Taq PCR Mix (Sigma). A 25µl reaction was set up containing 2µg cDNA template, 125ng of each primer, 12.5µl Red-Taq PCR Mix. A PCR program with the following parameters was used:

Cycle/s	Process	HIF1α
1	Pre-incubation (Initial denaturation)	95ºC, 5 minutes
35	Denaturation	95ºC, 1 minute
	Annealing	55ºC, 1 minute
	Extension	72ºC, 1 minute 30 seconds
1	Final extension	72ºC, 10 minutes

Table 2.10: Conditions for RT-PCR

2.2.25 PCR array

A Human Cell Motility RT^2 Profiler™ PCR Array was purchased from SABiosciences (a Qiagen company). This array was used to screen cDNA from cells treated with DMOG for 6 hours. The array was performed according to manufacturer's protocol on an ABI 7700 HT sequence detector (Applied Biosystems, Foster City, CA) using SYBR green technology (TebuBio, Peterborough, UK), with the program as follows:

Cycle/s	Process	
1	Pre-incubation	50ºC, 2 minutes
1	Initial denaturation	95ºC, 10 minutes
40	Denaturation	95ºC, 15 seconds
	Annealing	60ºC, 1 minute
	Extension	72ºC, 30 seconds

Table 2.11: Conditions for PCR Array

Statistical analyses were performed by comparing dCt values (housekeeping gene minus gene of interest) and analyzed using t-test. After combining the results from two independent experiments, the data were analyzed using the 2-ΔΔCt model normalized to the expression of housekeeping genes (B2M: Beta-2-microglobulin, HPRT1: Hypoxanthine phosphoribosyltransferase 1, RPL13A: Ribosomal protein L13a, ACTB: Actin, beta).

2.2.26 Statistical Analysis

Statistical significance was calculated using unpaired, two-tailed *t*-Test. Values were considered statistically significant if the P value was <0.05. For all figures, * indicates P value <0.05; ** indicates P value <0.005; and *** indicates P value <0.0005. Error bars represent SEM.

Chapter 3 - The characterization of invadopodia formation in response to HGF and EGF

Chapter 3 - The characterization of invadopodia formation in response to HGF and EGF

3.1 Introduction

3.1.1 Cancer cell invasion and invadopodia

A key feature of cancer is the ability of tumour cells to break through tissue barriers and invade into the underlying tumour stroma (Chambers et al., 2002). Highly invasive cancer cells cultured on physiological substrates form specialised membrane protrusions rich in actin filaments termed invadopodia. These structures have the capacity to degrade underlying matrix and are connected with high levels of proteolysis and cell signalling (Buccione et al., 2004). There is no specific marker for invadopodia, however, they are often highly enriched with actin filaments (F-actin) and components needed for actin assembly including the Arp2/3 actin nucleation complex, neural Wiskott Aldrich Syndrome protein (N-WASP) and cortactin (Artym et al., 2006; Buccione et al., 2004; Clark et al., 2007; Linder, 2007; Lorenz et al., 2004; Mizutani et al., 2002). A stepwise model for invadopodia formation has been proposed; however, the precise molecular mechanisms that govern the regulation of formation and dynamics of invadopodia are not well understood (Artym et al., 2006).

3.1.2 Epidermal and Hepatocyte Growth Factors (EGF)/ (HGF) in invadopodia formation

A large body of experimental evidence has demonstrated that HGF, c-Met, EGF and EGFR play critical roles in the motility and invasive growth of tumour cells, a hallmark of metastatic cancers (Comoglio and Trusolino, 2002; Maulik et al., 2002). EGF stimulation was shown to enhance invadopodia formation and ECM degradation in metastatic MTLn3 rat mammary adenocarcinoma cells, whereas inhibition of EGF receptor (EGFR) catalytic activity with the specific inhibitor AG1478 suppressed this effect (Yamaguchi et al., 2005). In addition to involvement in cell motility, HGF stimulation of endogenous c-Met receptors has also shown to promote invasion by enhancing the ability of cells to degrade matrix substrates

(Tague et al., 2004). Recently, HGF was shown to promote invadopodia formation in breast cancer cells which is dependent on Met activity (Rajadurai et al., 2012).

This chapter investigates the level of invadopodia formation in a panel of human cancer cell lines under basal growth conditions and in response to EGF/HGF stimulation.

3.2 Results

3.2.1 Screening of cancer cells for invadopodia formation

To study invadopodia formation, different cell lines were screened for their ability to degrade gelatin matrix. The aim was to identify a matched tissue pair of invasive and non-invasive cell lines. A panel of seven human cell lines were screened; highly metastatic melanoma, A375 cells were used as positive control for their well-established ability to form invadopodia (Ayala et al., 2008; Baldassarre et al., 2003); breast carcinoma, MDA-MB-231; and prostate carcinoma, PC3 cells and less invasive and non-metastatic colon carcinoma, HT29; breast carcinoma, MCF-7; and prostate carcinoma, DU145 cells. In order to determine the invadopodia forming potential of these cell lines, an *in vitro* fluorescent two-dimensional (2D) gelatin matrix degradation assay was employed (Artym et al., 2006).

There are a number of ways to quantify invadopodia depending upon image analysis procedures. The method employed in this study was to quantify the percentage of the cell population with invadopodia where they were defined as F-actin-rich puncta that co-localise with dark holes on the fluorescent gelatin **(figure 3.2 d)**. Under basal growth conditions, A375, MDA-MB-231 and PC3 cells form invadopodia **(figure 3.2 a to c)**, which is consistent with previous studies (Artym et al., 2006; Baldassarre et al., 2003; Desai et al., 2008). In contrast, no evidence of invadopodia formation was observed in the HT29, MCF-7 and DU145 non-invasive cells **(figure 3.4 a to c)**. The invadopodia forming potential of MCF10A cells, a non-tumorigenic human mammary epithelial cell line was also examined. These cells did not form invadopodia **(figure 3.4 d)**. This suggests a direct correlation between the ability to form invadopodia and metastatic potential of cancer cells.

3.2.2 EGFR and c-Met are expressed in a panel of six human cancer cell lines

Having identified those cells with basal invadopodia activity, the potential role of epidermal/hepatocyte growth factor (EGF)/ (HGF) stimulation was characterised. The endogenous expression levels of receptors for both ligands in these cell lines were initially established. Similar levels of EGFR were expressed in DU145, PC3 and MDA-MB-231 cells **(figure 3.1)**. HT29 cells express low levels of EGFR and

even less expression was observed in the A375 and MCF-7 cells **(figure 3.1)**. Similar levels of endogenous c-Met receptor expression were observed in DU145 and PC3, and also in MDA-MB-231 cells. HT29, A375 and MCF-7 cells express lower levels of c-Met **(figure 3.1)**.

3.2.3 EGF and HGF signalling can drive invadopodia formation

Having established that all cell lines express EGFR and c-Met, the ability of EGF and HGF to induce invadopodia formation was investigated. Initially, cells were maintained in 0.5% serum for 24 hours prior to growth factor stimulation. As expected, the percentage of A375, PC3 and MDA-MB-231 cells forming invadopodia was markedly reduced from above 30% in the basal growth condition to below 10% upon serum starvation **(figure 3.2d)**. During the analysis, F-actin puncta were observed to be present in some serum-starved cells where no gelatin degradation could be detected suggesting pre-invadopodia formation that is non-functional. Previous studies identified the actin-binding protein cortactin as indispensable for invadopodial actin core formation and cortactin has been proposed as an invadopodial molecular marker in addition to actin (Artym et al., 2006). Confocal microscopy analysis revealed that under basal growth conditions, F-actin puncta in the A375 melanoma and MDA-MB-231 carcinoma cells co-localized with cortactin and the degradation area on the gelatin matrix **(figure 3.3 a and b; top panel)** indicated by arrows. However, cortactin puncta was absent in cells grown under serum-deprived conditions where F-actin puncta were present and no gelatin degradation activity was observed **(figure 3.3 a and b; bottom panel)**, thus ruling out invadopodia formation in serum-starved cells.

Interestingly, following serum starvation, stimulation with EGF induced a significant increase in the percentage of PC3 and MDA-MB-231 forming invadopodia but not in A375 cells **(figure 3.2d)**. Furthermore, stimulation with HGF also showed a significantly increased invadopodia induction in A375, PC3 and MDA-MB-231 to almost the level of growing cells **(figure 3.2d)**. However, neither EGF nor HGF could promote invadopodia formation in the non-invasive cell lines **(figure 3.4e)**. Overall, HGF stimulation promoted a higher percentage of invadopodia formation than EGF.

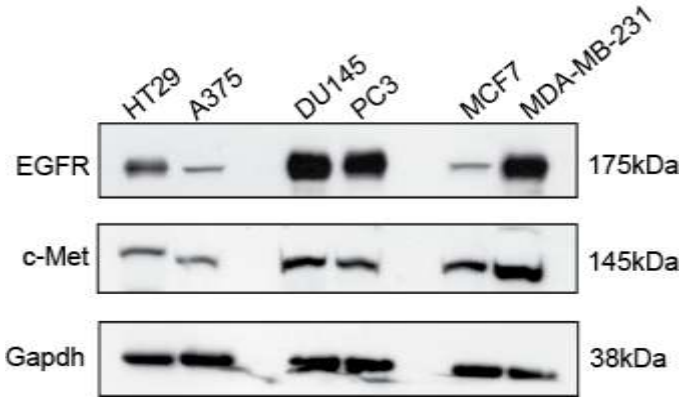

Figure 3.1 EGFR and c-Met are expressed in HT29, A375, DU145, PC3, MCF-7 and MDA-MB-231 cells. Whole cell lysates of growing cells were immunoblotted for EGFR and c-Met expressions. Gapdh, a house-keeping protein is used as a loading control. These results are representative of three independent experiments.

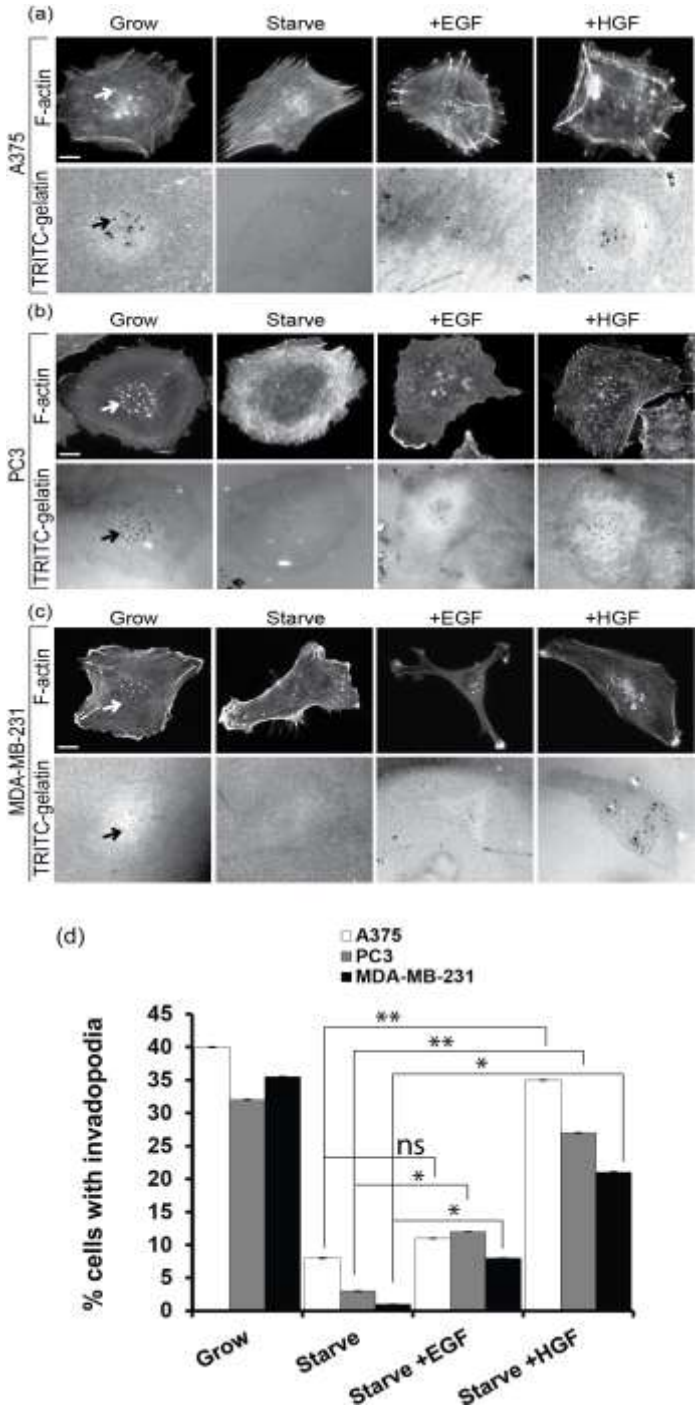

Figure 3.2 EGF stimulates invadopodia formation in PC3 and MDA-MB-231 cells but not in A375 cells. HGF stimulates invadopodia formation in A375, PC3 and MDA-MB-231 cells. (a-c) Cells were seeded onto TRITC-gelatin coated coverslips at $2X10^4$/ml, starved overnight and stimulated with EGF (100ng/ml) or HGF (10ng/ml). Cells were allowed to adhere and incubated at 37°C for 3 hours followed by fixation with 4% paraformaldehyde and stained for F-actin. Cells were imaged using fluorescence microscopy at X40 magnification. Black dots indicate areas where TRITC-gelatin was degraded by invadopodia (arrow). Scale bar=10μm. **(d)** Cells were scored for the presence of actin dots that co-localize with degradation on the gelatin and the mean percentage of cells with invadopodia formation calculated. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with starved was calculated using Student's *t*-test; *, P<0.05. **, P<0.005, ns=non-significant.

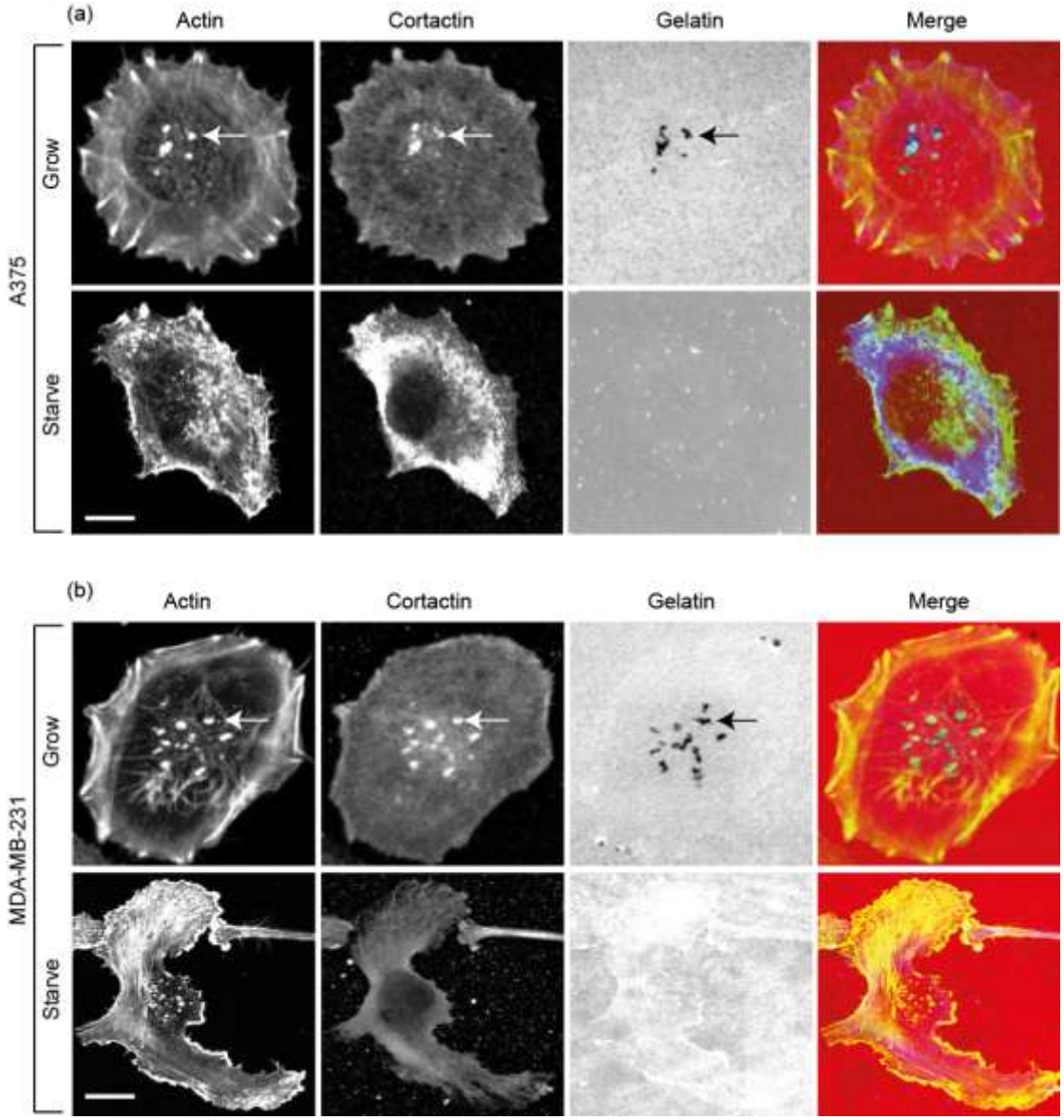

Figure 3.3 (a) A375 and (b) MDA-MB-231. Actin co-localizes with cortactin under basal growth conditions but not under serum deprived conditions. Cells were seeded onto TRITC-conjugated gelatin-coated coverslips at $2X10^4$/ml, allowed to adhere and incubated at 37°C for 3 hours followed by fixation with 4% paraformaldehyde. Cells were double stained for F-actin and cortactin. Cells were imaged using LSM 510 confocal microscopy at X63 magnification. Scale bar = 10µm.

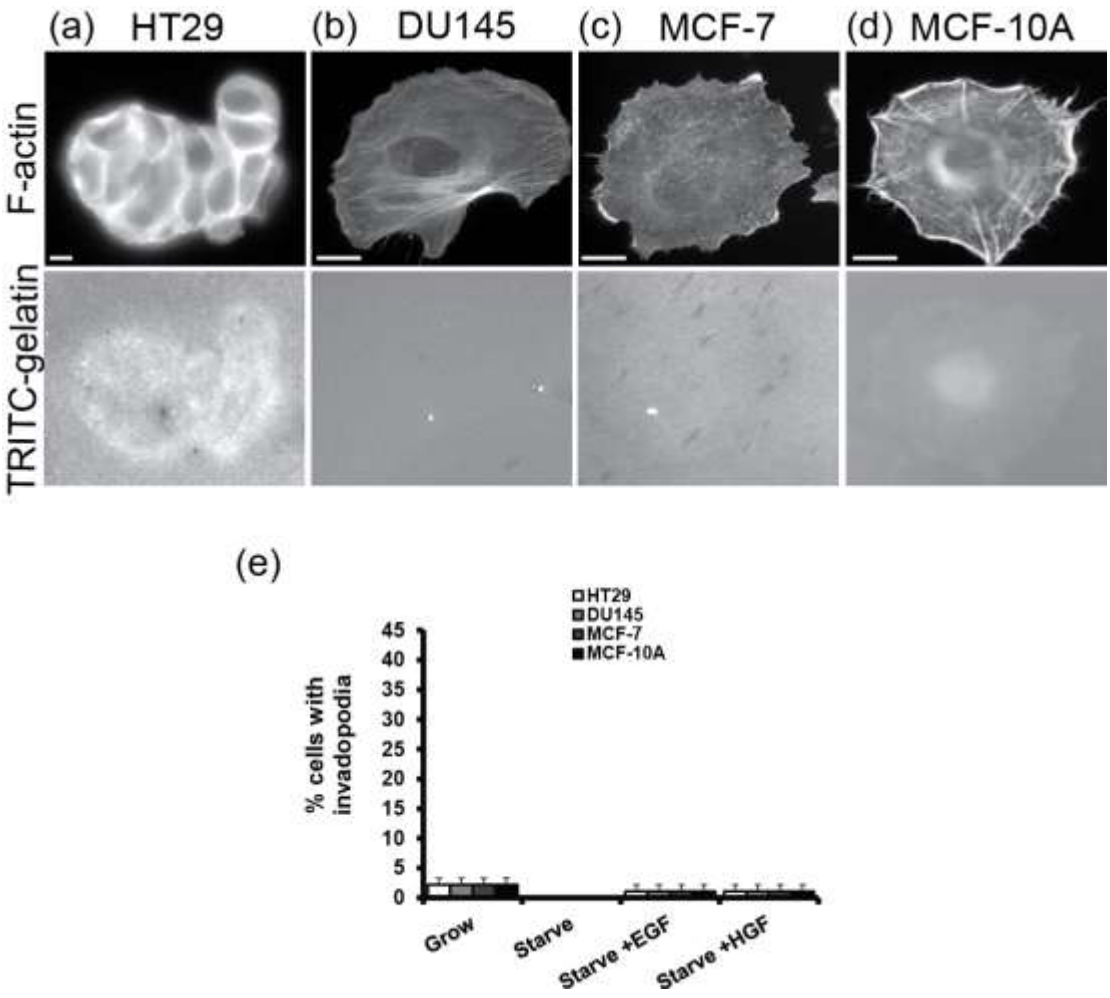

Figure 3.4 (a) HT29, (b) DU145, (c) MCF-7 and (d) MCF-10A cells are defective at forming invadopodia even under basal conditions. Cells were seeded onto TRITC-conjugated gelatin-coated coverslips at $2X10^4$/ml, allowed to adhere and incubated at 37°C for 3 hours followed by fixation with 4% paraformaldehyde and stained for F-actin. Cells were imaged using fluorescence microscopy at X40 magnification. Scale bar = 10µm.

3.2.4 Cells that do and do not form invadopodia are different in morphology

Often not all cells in a population will exhibit proteolytic behaviour, furthermore differences in cell morphology within the population seeded on gelatin could be observed. To further investigate these observations cell shape analysis using ImageJ software was performed to measure the elongation ratio and relative spread area of the invasive versus non-invasive cells and to test whether invadopodia forming cells have signature morphology. Interestingly invadopodia positive MDA-MB-231 cells were significantly less elongated than the invadopodia negative cells, whilst there was no significant difference between invadopodia positive and negative A375 cells **(figure 3.5b)**. In addition, both invadopodia positive A375 and MDA-MB-231 cells were significantly spread compared to the invadopodia negative cell populations **(figure 3.5a)**. This suggests that cell shape differs depending on whether cells are or are not forming invadopodia.

3.2.5 Gelatin degradation in EGF and HGF stimulated cells

Initial observations **(figure 3.2)** suggested that HGF not only induced the highest percentage of cells forming invadopodia but also induced increased underlying matrix degradation. To further investigate this observation, the degradation per cell area was quantified. As expected, there were trace amount of degradation observed in the starved cells **(figure 3.6 a and b; left panel)** consistent with their low ability to form invadopodia **(figure 3.2d)**. EGF stimulated cells showed more degradation than the starved cells **(figure 3.6)** and strikingly, both A375 and MDA-MB-231 cells stimulated with HGF significantly degraded more gelatin matrix compared to EGF **(figure 3.6 a and b; right panel)**. This indicates that HGF not only promotes invadopodia formation but also increases the ability of invasive cells to degrade gelatin matrix.

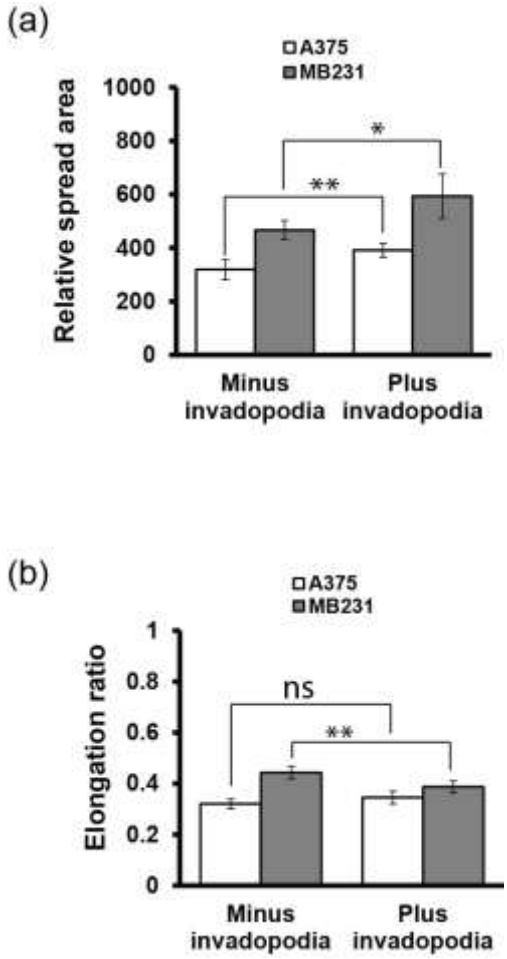

Figure 3.5 A375 and MDA-MB-231 cells that form invadopodia differ in morphology compared to those without invadopodia. Cells grown under basal conditions were seeded onto TRITC-conjugated gelatin-coated coverslips at $2X10^4$/ml. Images taken from fluorescence microscope were analysed for (a) relative spread area and (b) elongation ratio and quantified using ImageJ software. The results shown are mean ±SEM of 30 cells from each experimental condition over three independent experiments. Statistical significance compared to cells not forming invadopodia was calculated using Student's *t*-test; *, $P < 0.05$. **, $P < 0.005$, ns=non-significant.

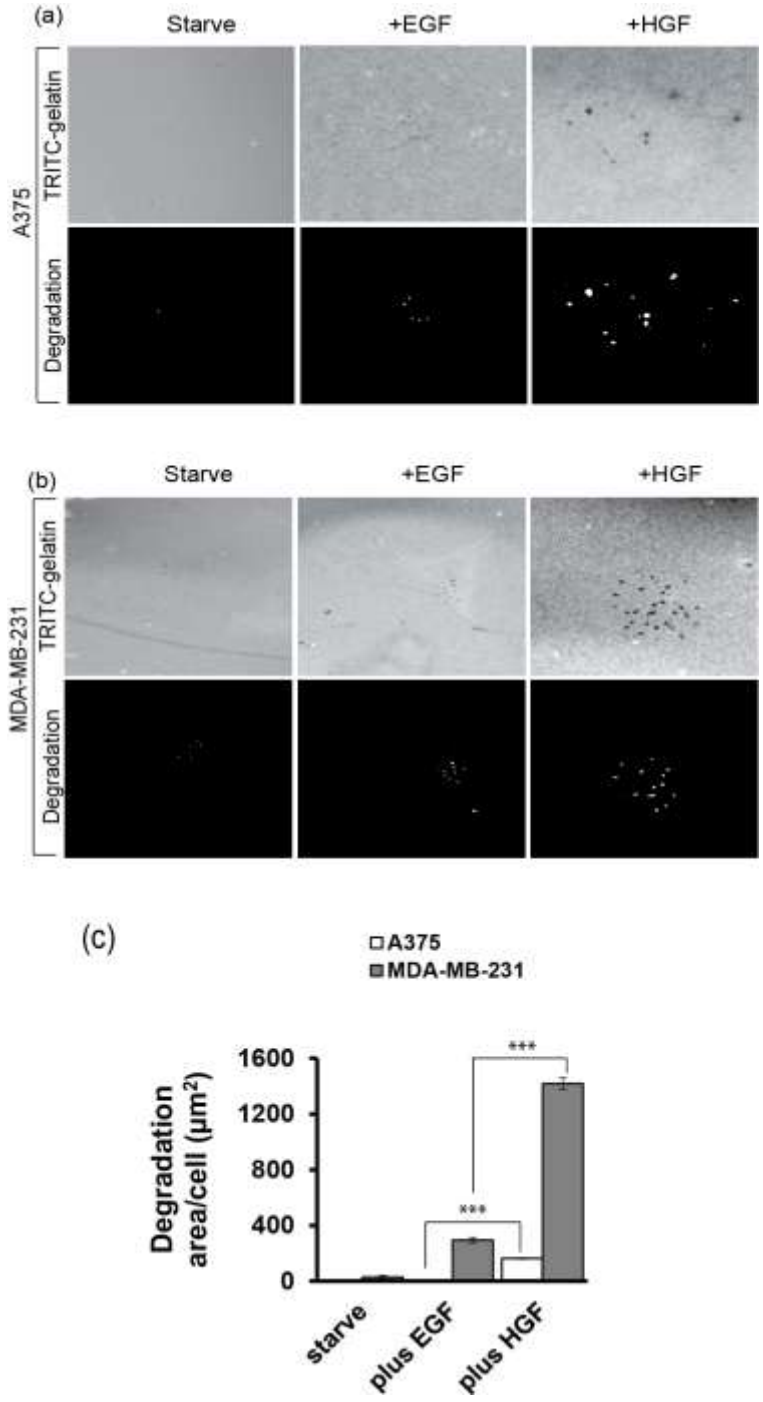

Figure 3.6 Gelatin degradation in EGF and HGF stimulated invadopodia forming cells. (a-b) Gelatin degradation assay was performed in starve, EGF and HGF treated A375 and MDA-MB-231 cells. This assay was done using ImageJ software. **(c)** The results shown are mean ±SEM of 30 cells from each experimental condition over three independent experiments. Statistical significance compared to cells not forming invadopodia was calculated using Student's *t*-test; ***, $P < 0.0005$.

3.3 Discussion

Invasive cancer cells can form a type of protrusive structure called invadopodia. These are actin-based membrane protrusions associated with ECM degradation, a critical step during cancer cell invasion (Murphy and Courtneidge, 2011). Considering that most tumour cells are surrounded by ECM *in vivo*, invadopodia are thought to be the protrusive structure formed and utilised by cancer cells in a physiological environment (Yamaguchi et al., 2005). Investigation of the molecular mechanisms involved in invadopodia formation and regulation are important for understanding tumour invasion and metastasis.

A strong connection between the invasive potential of cells and their ability to develop invadopodia and digest a fluorescently labelled gelatin matrix *in vitro* has previously been reported (Coopman et al., 1998). Recently, invasive carcinoma cells were shown to assemble invadopodia-like structures and invade into 3D matrix (Yu and Machesky, 2012). Additionally, mammary tumour cells have been suggested to form invadopodia *in vivo* (Gligorijevic et al., 2012).

In addition to Src-transformed fibroblasts, invadopodia have been reported in cell lines or primary tumour cells from malignant melanoma (e.g. LOX, RPMI7951), (Baldassarre et al., 2003; Mueller et al., 1999; Seals et al., 2005; Tague et al., 2004), breast/mammary carcinoma (e.g. MTLn3) (Artym et al., 2006; Hashimoto et al., 2004; Lorenz et al., 2004), and glioma (e.g. SNB19, U251MG) (Angers-Loustau et al., 2004; Chuang et al., 2004). Indeed, invadopodia may also be found formed by other malignant tumour cells.

In this chapter, seven cell lines were screened for invadopodia formation. In agreement with previous reports, the highly invasive MDA-MB-231, PC3 and A375 cells can form invadopodia **(figure 3.2)** (Artym et al., 2006; Baldassarre et al., 2003; Desai et al., 2008). However, the non-invasive MCF-7, DU145, and HT-29 cells did not form invadopodia, suggesting a direct correlation between the ability to form invadopodia and metastatic potential of cancer cells (Yamaguchi et al., 2005). Possibly, these non-invasive cells have a reduced ability to synthesize or secrete proteases, lack cell surface receptors required to localize the proteases, or are missing cytoskeletal components needed to stabilize protrusive invadopodia.

Additionally, these cells grow in colonies and form tight cell-cell junctions enriched in E-cadherin that can inhibit their invasive potential (Rajasekaran et al., 2001), whereas the invasive cells grow as single cells and can therefore respond independently to extracellular stimuli.

It is shown here that the percentage of highly invasive cells forming invadopodia markedly reduced upon serum starvation **(figure 3.2).** This is perhaps due to cells entering quiescent state (G0 phase) causing reduced production of natural growth factors or other molecular machineries required to sustain the invasive ability. A closer examination of some serum-starved cells within the population revealed that F-actin puncta were present in the absence of neither cortactin nor gelatin matrix degradation **(figure 3.3).** It has been reported that dendritic and bundled actin networks are first assembled at the ventral surface of the cell providing a core for invadopodia formation at pre-invadopodia or stage 0 (Schoumacher et al., 2010).

Invadopodia formation involves sequential stages of cortactin recruitment to membranes, matrix proteases accumulation, ECM degradation, and cortactin dissociation (Artym et al., 2006). Time-lapse imaging of invadopodia assembly revealed that cortactin induces formation of actin-rich invadopodia structures prior to recruitment of matrix proteases and subsequent matrix degradation (Artym et al., 2006). It is not well understood how cortactin is recruited to and its activity is regulated at invadopodia. Since cortactin can bind to several key components of invadopodia such as N-WASP and Arp2/3 complex, cortactin may cooperate with these proteins to assemble actin structures at invadopodia (Mizutani et al., 2002; Uruno et al., 2001). Therefore this suggests that serum-deprivation may only contribute to the induction of initial step that drives recruitment of invadopodia components to form pre-invadopodia F-actin structures. However, accumulation of these structures alone without recruitment of cortactin-dependent membrane proteases is insufficient to degrade the gelatin matrix.

During tumorigenesis, cellular growth, adhesion and motility become aberrantly activated leading to tissue invasion and metastasis. These can be driven by various signalling molecules such as growth factors. Studies have shown that stimulation with growth factors, such as platelet-derived growth factor (PDGF), and

transforming growth factor-β (TGFβ) induces invadopodia formation in cancer cells (Yamaguchi and Condeelis, 2007).

EGF receptor (EGFR) signalling is also known to play important role in the regulation of invadopodia formation. The most characterised role of EGFR signalling in invadopodia is its function upstream of Src activation and cortactin phosphorylation (Mader et al., 2011). EGF stimulation was reported to enhance invadopodia formation and ECM degradation in metastatic rat MTLn3 mammary carcinoma cells (Yamaguchi et al., 2005). In the current study, EGF was also shown to induce invadopodia formation in human MDA-MB-231 breast carcinoma cells **(figure 3.2).** Following on from the studies presented here, others have now reported that EGF can stimulate invadopodia formation in MDA-MB-231 cells (Pichot et al., 2010). Additionally, EGF can also increase the proteolytic activity of MDA-MB-231 cells (Busco et al., 2010). These data suggest that EGF is a potent stimulator of increased cell invasion and invadopodia formation not only in murine but also in highly invasive human breast carcinoma cells.

EGF was also able to induce invadopodia formation in PC3 prostate carcinoma cells **(figure 3.2),** an observation that has not been previously reported. Although, EGF has been shown to stimulate PC3 cell invasion in a micro invasion assay. This enhancement of invasion occurs in part by an overproduction of uPA, an extracellular protease important for matrix degradation (Jarrard et al., 1994). Conversely, EGF could not enhance invadopodia formation in human A375 melanoma cells likely to be as a result of low level of EGF receptor expression **(figure 3.1)**. In contrast, HGF stimulation enhanced invadopodia forming potential of human A375 melanoma cells. Similarly, HGF was shown to enhance the capacity of LOX human melanoma cells to degrade matrix substrates (Tague et al., 2004). HGF could also increase invadopodia formation in MDA-MB-231 and PC3 cells **(figure 3.2).**

Taken together, this study provides evidence that both HGF and EGF could promote invadopodia formation; however HGF was a more potent stimulator of gelatin degradation activity in comparison to EGF-induced invadopodia-forming cells **(figure 3.6).** This effect is interesting and presumably due to the contribution of HGF-induced signalling towards the increased production of matrix-

metalloproteinases (MMPs), key proteolytic proteins involved in the formation of invadopodia (Poincloux et al., 2009). HGF has been shown to actively promote production of MT1-MMP in MDA-MB-231cells, which further activated MMP2 and enhanced the invasiveness of breast cancer cells (Jiang et al., 2006). These proteins act as bridging molecules between matrix proteins, such as collagen and integrins facilitating matrix degradation (Poincloux et al., 2009). The underlying mechanism modulating this pathway remains elusive. Recently, c-Met was shown to localise at invadopodia in breast cancer cells and that formation of invadopodia relies on its activity (Rajadurai et al., 2012). However, it is not known if EGF receptors localize to invadopodia. Additionally, HGF and EGF have been demonstrated to stimulate divergent as well as redundant signal transduction pathways (Stolz and Michalopoulos, 1994), thus partly supports the data showing different effects of HGF and EGF on inducing invadopodia and gelatin matrix degradation.

EGF and HGF stimulation could not induce invadopodia in the non-invasive cell lines. HGF has a well-recognised role in promoting EMT by inducing cell scattering in MDCK (Balkovetz, 1998; Stoker and Perryman, 1985) and in DU145 colony forming cells (Bright et al., 2009; Wells et al., 2005). Data presented here would suggest that growth factor stimulation alone may be insufficient to facilitate the complete transition from a non-invasive to invasive phenotype with matrix degrading capacity. Perhaps for non-invasive cells to be able to form invadopodia and degrade matrix, they require neoplastic transformation by over-expression of oncogenic proteins such as Src to acquire invasive capacity (Chen, 1989).Cell shape analysis to test whether invadopodia forming cells have signature morphology revealed that invadopodia forming cells were more spread in both cell lines **(figure 3.5).** This could indicate the activation of integrin-mediated adhesion events at cell-matrix sites that triggers the assembly of invadopodia to facilitate movement and penetration of cell body to degrade matrix (Poincloux et al., 2009). One interesting possibility is that EGFR and c-Met may induce invadopodia via cross-talk with cell surface integrins (Ivankovic-Dikic et al., 2000).

This chapter describes the effect of extracellular stimulation by EGF or HGF on invadopodia formation across a panel of cell lines. Following detailed analysis of invadopodia forming potential, two cell lines were selected for further study. MDA-MB-231 cells exhibited significant invadopodia formation that could also be

substantially reduced by serum starvation and elevated by stimulation with both growth factors. MCF-7 cells were chosen as a complimentary tissue matched cell line as these cells had no significant invadopodia forming activity regardless of culture conditions.

Chapter 4 - The characterization of invadopodia formation in response to hypoxia

Chapter 4 - The characterization of invadopodia formation in response to hypoxia

4.1 Introduction

Chapter 3 demonstrated that invadopodia formation could be induced by growth factor stimulation. However, during tumour progression, other environmental cues are also received by a cancer cell. One such cue is a change in the oxygen levels surrounding the tumour mass. Typically the local vasculature is unable to supply a sufficient amount of oxygen and nutrients to the rapidly growing tumour. This results in intratumoral hypoxia (Semenza, 2002; Vaupel, 2004). To resist the negative effects of hypoxia, tumour cells increase the expression of the α-subunit of HIF-1 which is largely known to promote angiogenesis (Harris, 2002; Semenza, 2003; Vaupel, 2004). In some breast carcinomas, HIF-1α is over-expressed and has been associated with increased blood vessel density, tumour aggressiveness and poor prognosis (Bos et al., 2003; Kimbro and Simons, 2006; Vleugel et al., 2005). However, more recent studies also suggest that hypoxia can promote cancer cell invasion (Kimbro and Simons, 2006; Lu and Kang, 2010).

Matrigel invasion assays using Boyden chambers demonstrated that hypoxia promotes invasion in breast carcinoma cells via HIF-1α over-expression (Choi et al., 2011). Recently, hypoxia has been shown to influence invadopodia formation in human fibrosarcoma cells (Lucien et al., 2011). However a direct effect of HIF-1α over-expression in the formation of invadopodia has not been investigated.

This chapter investigates the invadopodia forming potential of the chosen tissue-matched cell lines MDA-MB-231 and MCF-7 in response to hypoxia by inducing increased expression of HIF-1α protein. Furthermore, to compliment chapter 3 studies, the synergistic effect of hypoxia in combination with HGF stimulation was also examined.

4.2 Results

4.2.1 HIF-1α expression is maintained for up to 6 hours with DMOG

In order to investigate the effects of hypoxia on the invasive behaviour of the two chosen breast cancer cell lines, the initial approach was to chemically induce HIF-1α expression with dimethyloxaloylglycine (DMOG) at a concentration of 0.5mM. DMOG, routinely used as a hypoxia mimetic, stabilizes HIF-1α protein by inhibiting prolyl and asparaginyl hydroxylases under normal oxygen tension (Marchbank et al., 2011). The expression level of HIF-1α upon DMOG treatment over a period of 6 hours was compared to the basal level in both cell lines. Endogenous HIF-1α expression level was significantly increased **(figure 4.1a and b)** and can be observed as early as 2 hours following DMOG treatment in MDA-MB-231 and MCF-7 cells. As expected, a weak basal level of HIF-1α expression was observed in the MDA-MB-231 cells **(figure 4.1a)** whilst the MCF-7 cells do not detectably express HIF-1α under basal growth conditions **(figure 4.1b)**. The expression level of a closely related family member HIF-2α was also determined. Whilst both MDA-MB-231 and MCF-7 cells express HIF-2α under basal conditions **(figure 4.1c),** there was no significant increase in the levels of HIF-2α observed in the MDA-MB-231 cells upon 6 hours DMOG treatment **(figure 4.1c)**.

4.2.2 DMOG increases invadopodia formation and gelatin degradation

Having successfully induced HIF-1α over-expression in the two cell lines, the effect on invadopodia forming potential was examined. In the non-treated cell population, 37% of the MDA-MB-231 cells form invadopodia **(figure 4.2 a left panel and b).** When the cells were treated with DMOG, the percentage of cells forming invadopodia significantly increased to 50% **(figure 4.2 a middle panel and b).** The effect of maintaining DMOG presence during the gelatin degradation assay was next tested. Interestingly, when more DMOG was added to cells, a further increase in the percentage of invadopodia to 67% was observed **(figure 4.2 a right panel and b).** These results indicate that increased HIF-1α expression correlates with increased ability of these cells to form invadopodia.

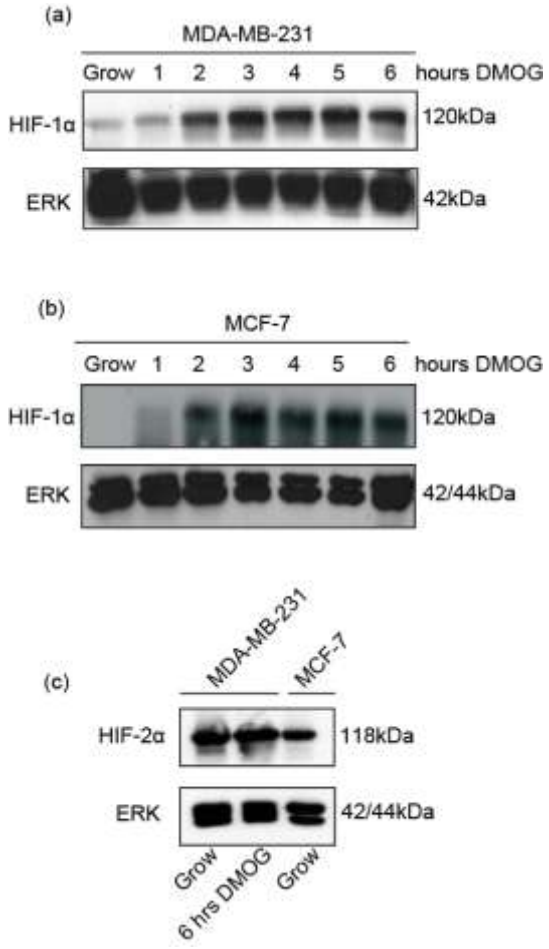

Figure 4.1 Increased HIF-1α expression is maintained for up to 6 hours upon DMOG stimulation. **(a)** MDA-MB-231 and **(b)** MCF-7 cells were seeded at $3X10^4$ cells/ml and allowed to adhere for 24 hours. Cells were stimulated with (0.5mM) dimethyloxaloylglycine (DMOG) for up to 6 hours. Cells were lysed for 10 minutes in lysis buffer (see materials and methods). Equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of HIF-1α (Cell Signalling Technology) and **(c)** HIF-2α (Santa Cruz) antibodies. ERK was used as a loading control. These results are representative of three independent experiments.

Given that HGF also promoted invadopodia formation in these cells, HGF was also added to the DMOG treated cells seeded on TRITC-gelatin for 3 hours to determine whether there was a synergistic effect on invadopodia formation. However, no significant increase in the percentage of invadopodia was observed when the cells were stimulated with HGF compared to the cells stimulated with DMOG alone **(figure 4.2b).** In addition to its effect on increasing invadopodia forming potential, cells pre-treated with DMOG for 6 hours exhibited an increase in degradation activity in comparison to the untreated cells **(figure 4.2 a bottom panel and c)**. Furthermore, cells with 6 hours pre-treatment followed by further addition of 3 hours DMOG enhanced gelatin degradation in comparison to the other two conditions **(figure 4.2 a bottom right panel and c).** These results indicate that increased HIF-1α expression does not only influence invadopodia forming potential but also increased gelatin matrix degradation activity of the MDA-MB-231 cells.

4.2.3 DMOG does not induce invadopodia formation in MCF-7 cells

MCF-7 cells do not form invadopodia even under basal growth conditions **(figure 3.4c)**. Furthermore, HGF could not induce invadopodia formation in these cells **(figure 3.4e).** No evidence of invadopodia formation in MCF-7 cells was observed **(figure 4.3 f)** following DMOG treatment for 6 hours. In parallel with DMOG-only treatment, maintaining the presence of DMOG for 3 hours and overnight was performed. This still resulted in no induction of invadopodia formation in these cells **(figure 4.3 f).** Additionally, stimulation with HGF for 3 hours could not induce invadopodia in the DMOG-treated cells **(figure 4.3 f).**

Under basal/non-treated conditions, the MCF-7 cells grow in colonies **(figure 4.3 a left panel)**, however following 6 hours DMOG treatment, some of these cells appeared to start dissociating from their colonies **(figure 4.3 b middle panel).** This cell:cell dissociation observation was quantified using an established protocol (Fram et al., 2011). The percentage of cells attempting to scatter from colonies significantly increased in response to DMOG treatment compared to untreated cells **(figure 4.3 g).** Moreover, maintaining DMOG for 3 hours and overnight further enhanced this effect **(figure 4.3 g).** A combined HGF stimulation in DMOG-treated cells also increased the percentage of cells attempting to scatter compared to DMOG stimulation alone **(figure 4.3 g)** suggesting an additive effect.

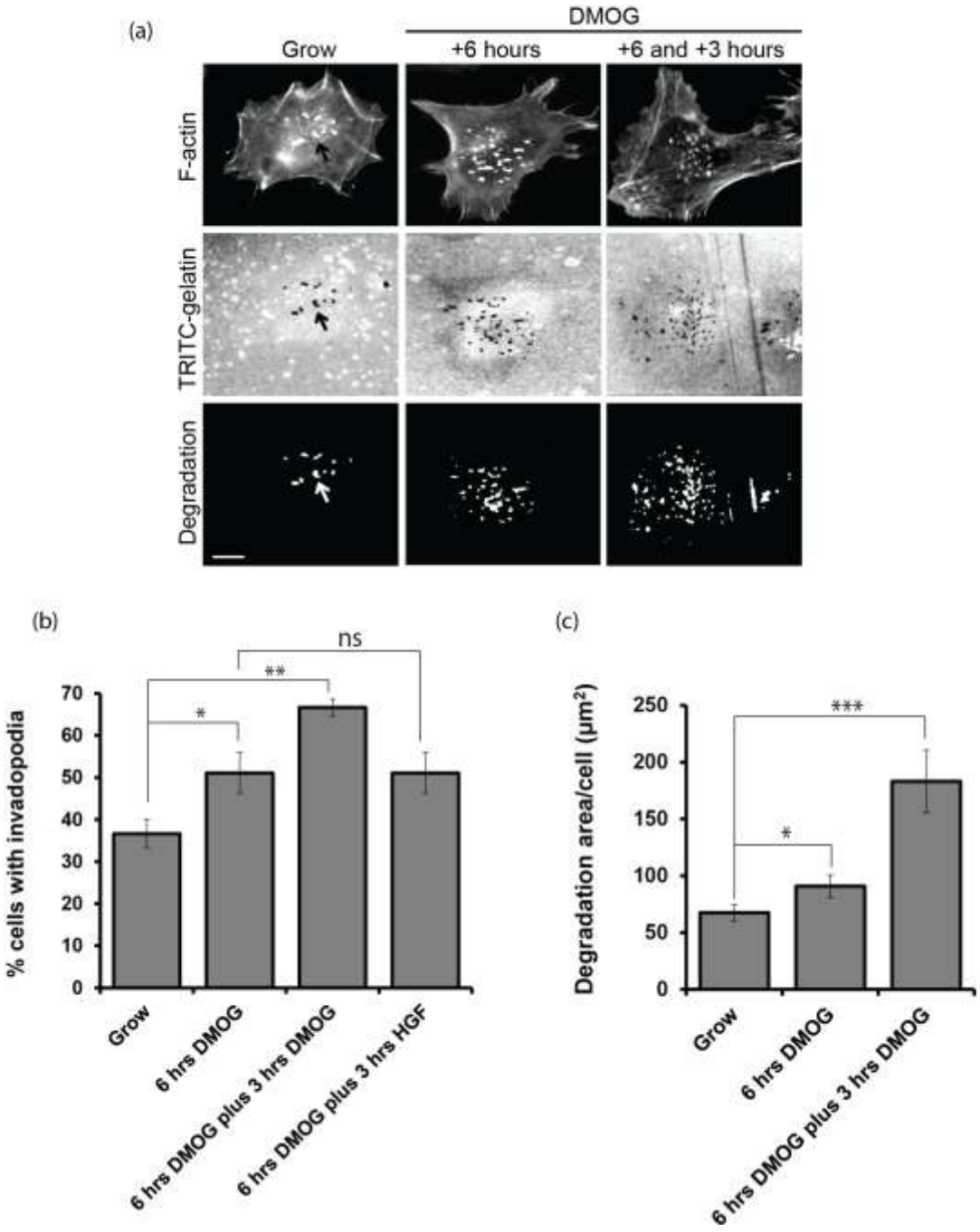

Figure 4.2 DMOG increases invadopodia formation and gelatin degradation in MDA-MB-231 cells. (a) Cells were seeded at $3X10^4$ cells/ml in a 6-well plate and then stimulated with (0.5mM) DMOG for 6 hours. Cells were dissociated using non-enzymatic cell dissociation buffer and re-seeded on gelatin-coated coverslips at $2X10^4$ cells/ml before being allowed to adhere for 3 hours at 37°C. Cells were then fixed with 4% PFA, stained for F-actin using Phalloidin 488 (Invitrogen) and imaged using fluorescence microscopy. **(b)** Cells were scored for the presence of actin puncta that colocalize with an area of degradation on the gelatin (arrow) and the mean percentage of cells with invadopodia formation calculated. Scale bar=10µm. **(c)** Gelatin degradation assay was performed with all three conditions. This assay was done using ImageJ software. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with grow was calculated using Student's *t*-test; *, P<0.05. **, P<0.005. ***, P<0.0005, ns= non-significant.

The cells that were attempting to scatter exhibited altered morphology in response to 6 hours DMOG treatment, maintaining DMOG presence for 3 hours and overnight, and also stimulation with HGF **(figure 4.3b-e).** This phenotype was quantified using ImageJ cell shape analysis. DMOG-treatment significantly reduced cell spread area and cells became markedly elongated **(figure 4.3 h and i).** These results indicate that although DMOG could not induce invadopodia formation, it could promote cell:cell dissociation and affect cell morphology.

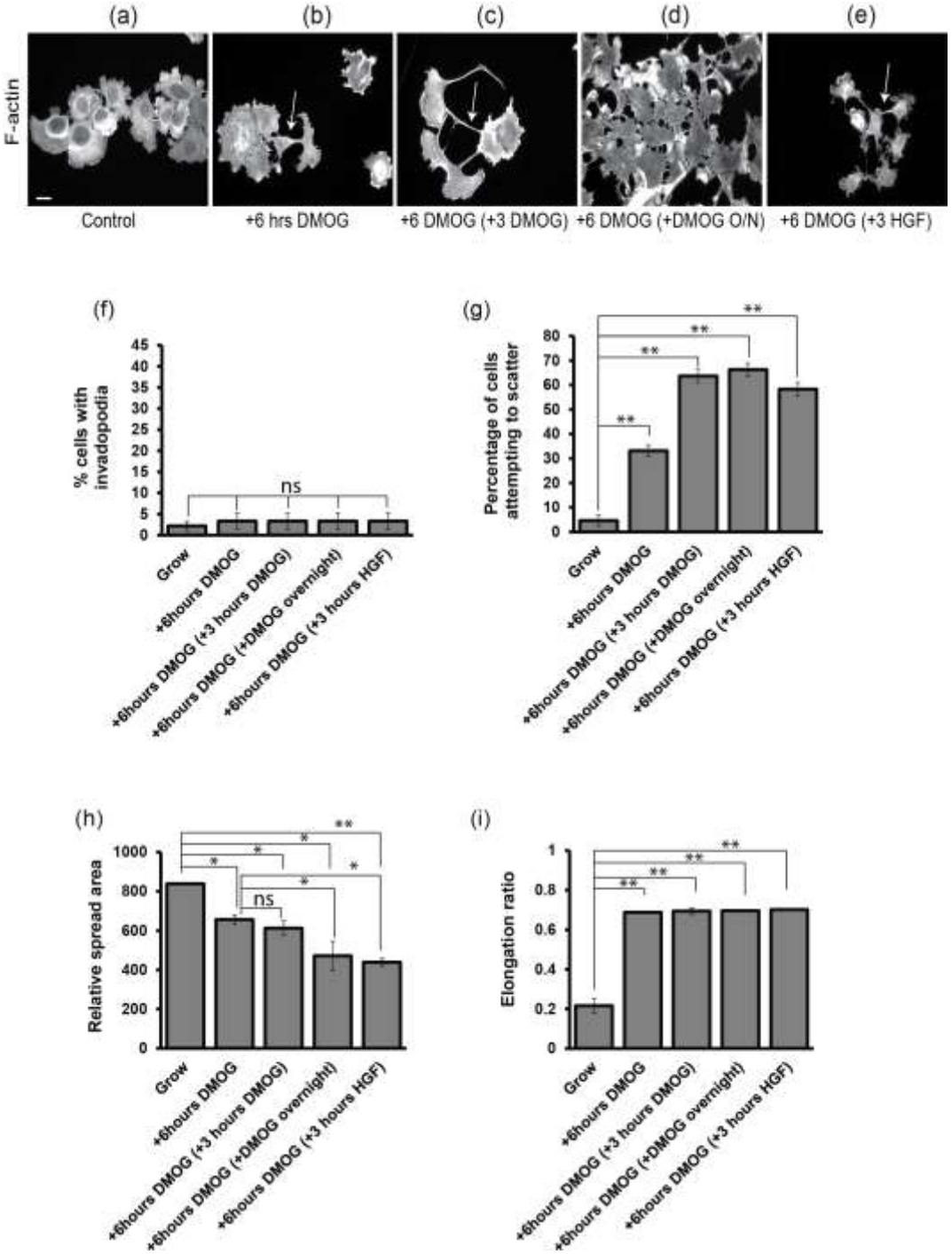

Figure 4.3 DMOG does not induce invadopodia formation in MCF-7 cells but reduced cell-cell contact between cells in colonies. (a) MCF-7 cells were seeded at $3X10^4$ cells/ml in a 6-well plate. Cells were then stimulated with DMOG for 6 hours **(b).** Cells were dissociated using non-enzymatic cell dissociation buffer. Cells were then counted and re-seeded on gelatin-coated coverslips at $2X10^4$ cells/ml with **(c)** additional DMOG for 3 hours, **(d)** additional DMOG overnight and **(e)** stimulation with HGF and allowed to adhere for 3 hours at 37°C. Cells were then fixed with 4% PFA, stained for F-actin using Phalloidin 488 (Invitrogen) and imaged using fluorescence microscopy. **(f)** Mean percentage of invadopodia. **(g)** Mean percentage of cells attempting to scatter was calculated. **(h-i)** ImageJ software was used to analyse cell area and elongation ratio of cells. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with grow was calculated using Student's *t*-test; *, P<0.05. **, P<0.005, ns=non-significant.

4.2.4 Loss of HIF-1α expression attenuated DMOG response

Results presented here **(figure 4.2)** suggest that increased HIF-1α expression leads to increased invadopodia forming potential **(figure 4.2a-c).** To rule out any off target effects of the compound, and to establish whether the DMOG-induced phenotype was a direct result of specific stabilization of HIF-1α expression, a HIF-1α depletion experiment was performed in the background of DMOG treatment. As shown in **(figure 4.4a and b)**, 80% of HIF-1α protein expression was successfully depleted, with no detectable change in the expression of HIF-2α **(figure 4.4a)**. Having successfully knocked-down HIF-1α expression, cells were treated with DMOG for 6 hours then seeded on TRITC-gelatin coverslips for 3 hours **(figure 4.4c).** Cells transfected with control siRNA form invadopodia **(figure 4.4c and d),** consistent with previous results **(figure 4.2)** whilst DMOG treated cells with reduced HIF-1α expression had a significantly reduced response **(figure 4.4c and d).**

4.2.5 GFP- HIF-1α is localised in the nucleus and functional

To complement the DMOG studies and further investigate the specific role of HIF-1α, an over-expression system was employed. Using Gateway Cloning Technology, a GFP-tagged version of HIF-1α was generated. Under normal oxygen conditions, HIF-1α is rapidly degraded. Thus the stability of GFP-HIF-1α was tested in HEK-293 cells; as these cells have high transfection efficiency. A 72-hour time course experiment was performed to examine GFP-HIF-1α expression, stability and localisation. HIF-1α was successfully over-expressed in HEK-293 cells **(figure 4.5a)** and expression was maintained for up to 72 hours **(figure 4.5b).** Additionally HIF-1α was correctly localised in the nucleus **(figure 4.5a)**.

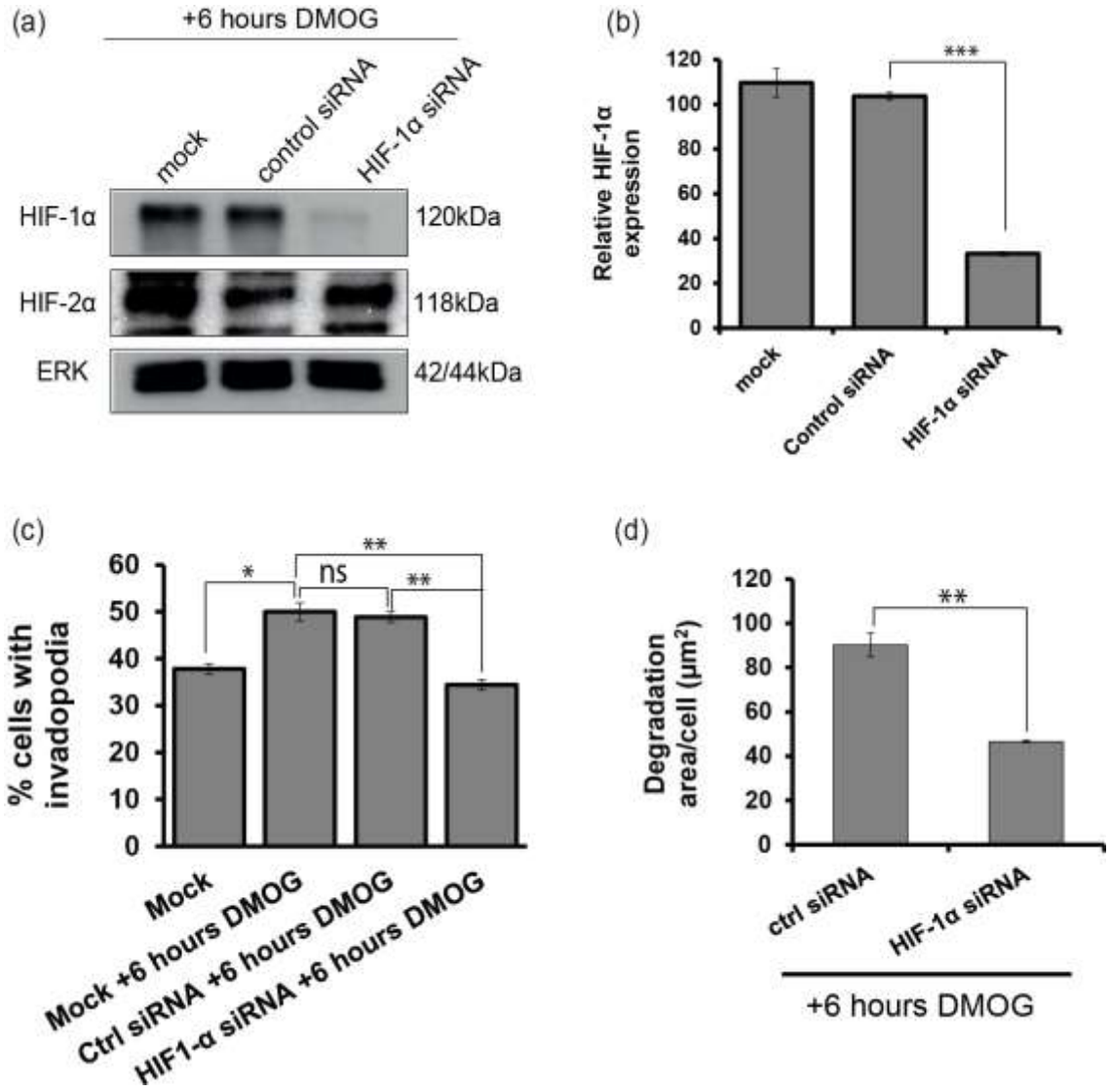

Figure 4.4 Loss of HIF-1α expression attenuated DMOG response. (a) MDA-MB-231 cells were seeded at $3X10^4$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with mock, control and HIF-1α siRNAs for 48 hours, followed by stimulation with DMOG for 6 hours. Cells were lysed for 10 minutes in lysis buffer (see materials and methods). Equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of HIF-1α (Cell Signalling Technology). Membrane was stripped and re-probed for levels of HIF-2α (Santa Cruz). ERK, a house-keeping protein was used as a loading control. **(b)** Relative expression of HIF-1α was measured using densitometric analysis. **(c)** HIF-1α KD cells were seeded on gelatin-coated coverslips. **(d)** Cells were scored for the presence of actin puncta that colocalize with area of degradation on the gelatin (arrow) and the mean percentage of cells with invadopodia formation calculated. Scale bar=10μm. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with grow was calculated using Student's *t*-test; *, P<0.05. **, P<0.005. ***, P<0.0005, ns=non-significant

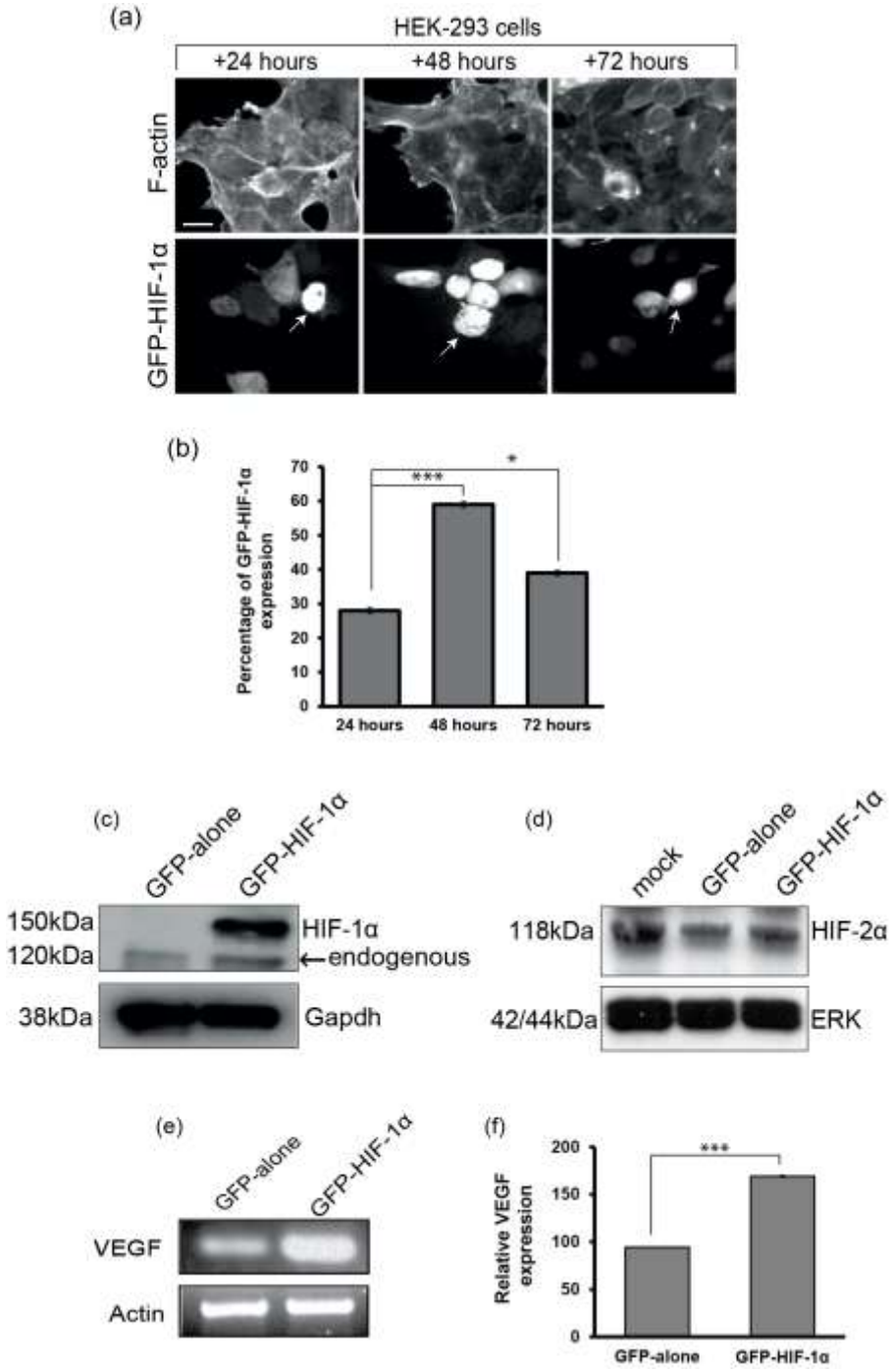

Figure 4.5 GFP-HIF-1α is localised in the nucleus and functional. **(a)** HEK-293T cells were seeded at $1X10^5$ cells/ml on coverslips in a 6-well plate and transfected with GFP-HIF-1α. Cells were fixed at 24, 48 and 72 hours following transfections with 4% PFA, stained for F-actin using TRITC-Phalloidin (Invitrogen) and imaged using fluorescence microscopy. **(b)** Cells were scored for the presence of GFP-HIF-1α expression in the nucleus at indicated time points (arrow). **(c)** MDA-MB-231 cells were seeded at $1X10^5$ cells/ml in a 6-well plate and transfected with GFP-HIF-1α using Lipofectamine 2000. Cells were lysed at 24 hours following transfections. Western blot of the lysates was probed for levels of HIF-1α (Cell Signaling Technology) and **(d)** HIF-2α (Santa Cruz). ERK and gapdh, are used as loading controls**. (e)** RT-PCR of MDA-MB-231 cells transfected with GFP-control and GFP-HIF-1α using VEGF specific primers. **(f)** Relative expression of VEGF was measured using densitometric analysis. Scale bar=10μm. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with 24 hours was calculated using Student's *t*-test; *, P<0.05. ***, P<0.0005.

Subsequently, GFP-HIF-1α over-expression was tested in the breast cancer cell lines. HIF-1α over-expression was stable under normal oxygen tension **(figure 4.5)** and western blot analysis confirmed the size and stability of over-expressed HIF-1α in both cell lines at 150kDa **(figure 4.6a and 4.7a).** The expression level of a closely related family member HIF-2α was also determined. There was no change in the expression of HIF-2α in the presence of over-expressed GFP-HIF-1α **(figure 4.6b).** The ability of GFP-HIF-1α to function normally in cells was also tested by monitoring the level of VEGF mRNA expression in control and transfected cells. HIF-1α is known to induce a large increase in VEGF expression and this increase was detected in MDA-MB-231 cells expressing GFP-HIF-1α compared to control cells expressing GFP-alone **(figure 4.6 c and d).**

4.2.6 GFP-HIF-1α over-expression increases invadopodia formation and gelatin degradation

Having established that GFP-HIF-1α is stable and functional when overexpressed in breast cancer cells, overexpressing cells were tested for invadopodia forming activity. GFP-HIF-1α or control GFP alone transfected cells were seeded on TRITC-gelatin coverslips for 3 hours and assayed for invadopodia formation **(figure 4.6e and 4.7b).** Over-expression of GFP-HIF-1α significantly increased the percentage of cells with invadopodia in the MDA-MB-231 cells compared to GFP-alone **(figure 4.6e).** Additionally, GFP-HIF-1α over-expressing cells also had an increased ability to degrade gelatin matrix **(figure 4.6e right panel).** The level of elevation was consistent with the level of DMOG-induced invadopodia formation and gelatin degradation **(figure 4.2).** In contrast, MCF-7 cells exhibited no induction of invadopodia in response to over-expression with GFP-HIF-1α **(figure 4.7 b and c).**

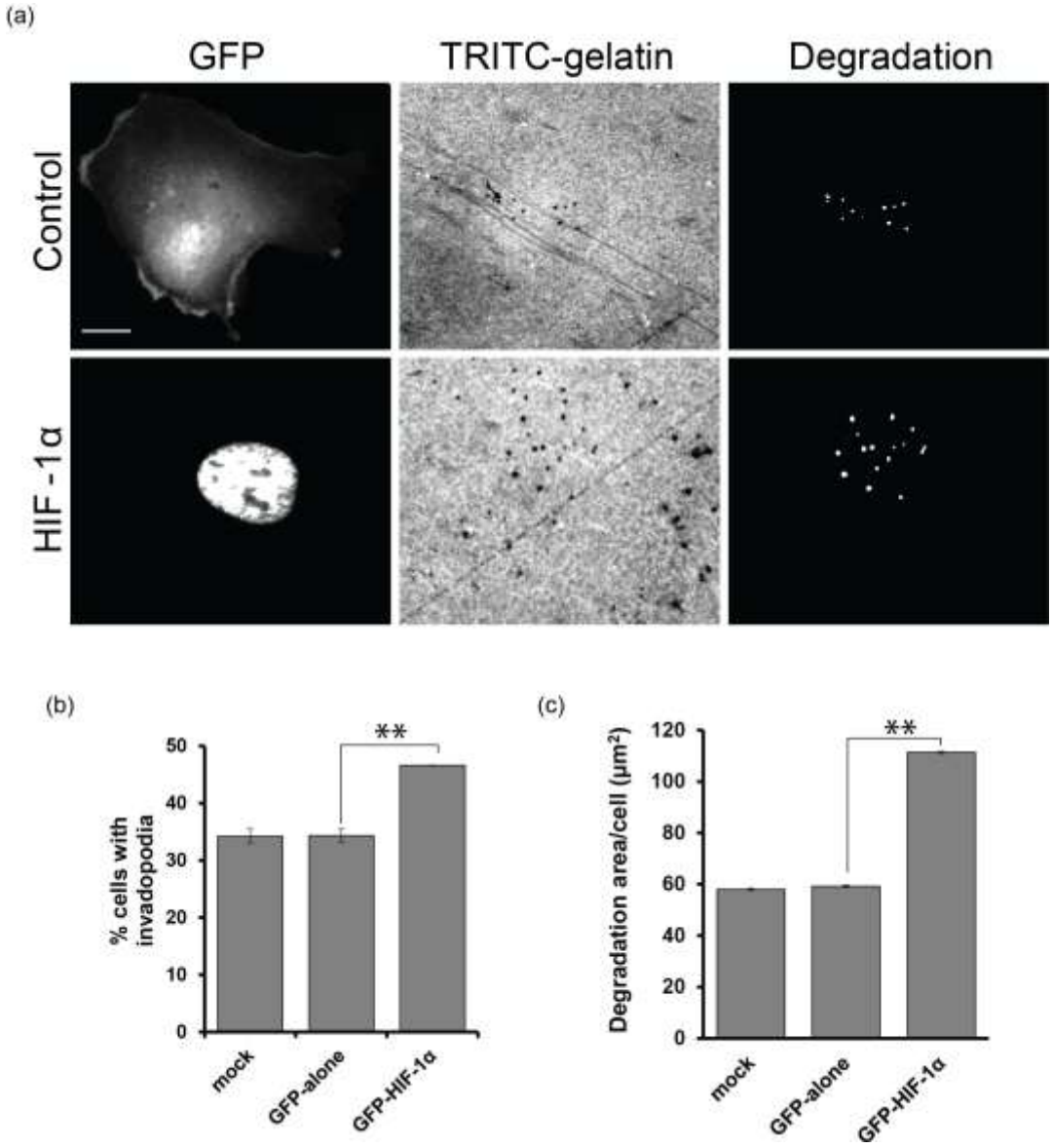

Figure 4.6 GFP-HIF-1α over-expression induces invadopodia formation and gelatin degradation in MDA-MB-231 cells. **(a)** GFP-control and GFP-HIF-1α cells were seeded on gelatin coverslips for 3 hours. **(b)** Cells were scored for the presence of GFP-HIF-1α expression in the nucleus and corresponding actin puncta that colocalize with area of degradation on the gelatin. Scale bar=10μm. **(c)** ImageJ software was used to quantify the area of degradation per cell area. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with GFP-control was calculated using Student's *t*-test; **, P<0.005.

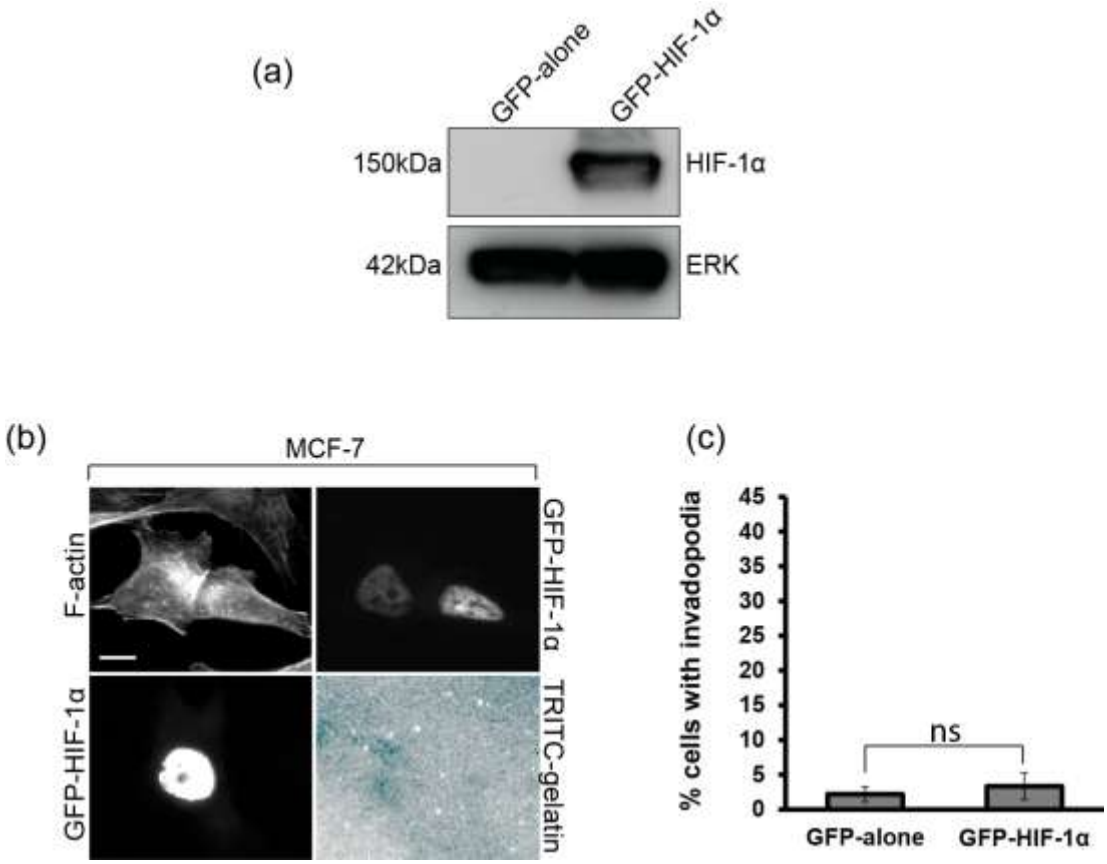

Figure 4.7 GFP-HIF-1α over-expression does not induce invadopodia formation in MCF-7 cells. (a) MCF-7 cells were seeded at $1X10^5$ cells/ml in a 6-well plate and transfected with GFP-HIF-1α using Lipofectamine 2000. Cells were lysed at 24 hours following transfections. Western blot of the lysates was probed for levels of HIF-1α (Cell Signalling Technology). ERK, a house-keeping protein was used as a loading control **(b)** Cells were dissociated using non-enzymatic cell dissociation buffer. Cells were then counted and re-seeded on gelatin-coated coverslips at $2X10^4$ cells/ml. **(c)** Cells were scored for the presence of GFP-HIF-1α expression in the nucleus and corresponding actin puncta that colocalize with area of degradation on the gelatin. Scale bar=10µm. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with GFP-control was calculated using Student's *t*-test; ns= non-significant.

4.2.7 GFP-HIF-1α over-expression increases cell: cell dissociation

DMOG treatment has revealed a significant induction of cell: cell detachment from tight colonies in MCF-7 cells **(figure 4.3).** Interestingly, GFP-HIF-1α over-expression in MCF-7 cells also exhibited a similar phenotype **(figure 4.8d).** To verify this effect, E-cadherin, a well-established epithelial marker was used to label cell: cell junctions in GFP-HIF-1α over-expressing cells. E-cadherin staining was specifically reduced in cells over-expressing GFP-HIF-1α particularly at junctions **(figure 4.8c).** In parallel, western blot analysis revealed an approximately 3-fold reduction in E-cadherin protein expression levels **(figure 4.8a and b).**

4.2.8 Hypoxic environment increases gelatin degradation

Increased HIF-1α expression by either DMOG or GFP-HIF-1α promotes invadopodia formation in MDA-MB-231 cells **(figure 4.2 and 4.6**). However, it is important to be able to demonstrate the effects of increased HIF-1α expression on invadopodia formation under more physiological conditions of oxygen deprivation. To do this, a hypoxia modulator incubator was utilised. Cells were incubated either under normoxic or hypoxic environment with either 20% or 1-1.5% oxygen supply respectively. This method is a physiological approach used in numerous hypoxia studies to observe different effects on cells (Fujiwara et al., 2007; Lee et al., 2010; Lu et al., 2010). HIF-1α levels drop rapidly when cells were removed from the incubator, thus the gelatin degradation assay was slightly modified to accommodate this effect. MDA-MB-231 cells were seeded onto TRITC-gelatin coverslips followed by incubation in a normoxia or hypoxia incubator for 6 hours. An additional time point of 16 hours was also chosen. In parallel, cells were seeded on plastic for 6 or 16 hours in normoxic or hypoxic condition for extraction of protein lysates to assay for levels of HIF-1α expression. Endogenous HIF-1α expression level was significantly increased when cells were incubated in hypoxic conditions **(figure 4.9a)** but HIF-2α expression remained unchanged **(figure 4.9a).** Interestingly, cells incubated for 6 and 16 hours under hypoxic conditions significantly degraded more gelatin than normoxic conditions **(figure 4.9b and c)**. These results indicate that hypoxia has a significant effect on tumour cell behaviour, in part by increasing invasive potential.

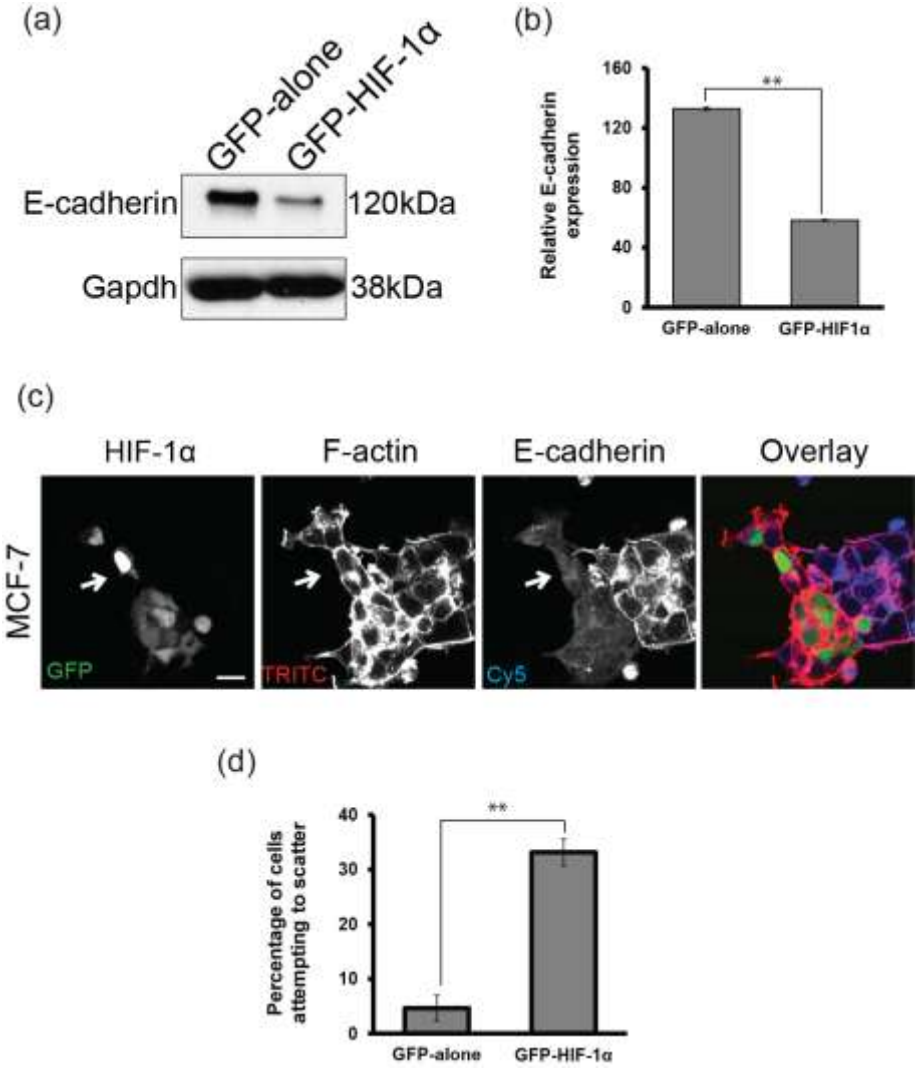

Figure 4.8 GFP-HIF-1α over-expression reduces E-cadherin expression and promotes cell: cell dissociation of MCF-7 cells. (a) MCF-7 cells were seeded at $1X10^5$ cells/ml in a 6-well plate and transfected with GFP-HIF-1α using Lipofectamine 2000. Cells were lysed at 24 hours following transfections. Western blot of the lysates was probed for levels of E-cadherin (Cell Signaling Technology). Gapdh, a house-keeping protein was used as a loading control **(b)** Densitometric analysis of E-cadherin protein expression. **(c)** Cells were dissociated using non-enzymatic cell dissociation buffer. Cells were then counted and re-seeded on glass coverslips at $2X10^4$ cells/ml. Cells were then fixed with 4% PFA, stained for F-actin using Phalloidin 488 (Invitrogen) and E-cadherin and imaged using confocal microscopy. **(d)** Mean percentage of GFP-HIF-1α over-expressing cells attempting to scatter was calculated. The results shown are mean value ±SEM of 30 cells from each experimental condition over two separate experiments. Scale bar=10μm. Statistical significance compared with GFP-alone was calculated using Student's *t*-test; **, P<0.005.

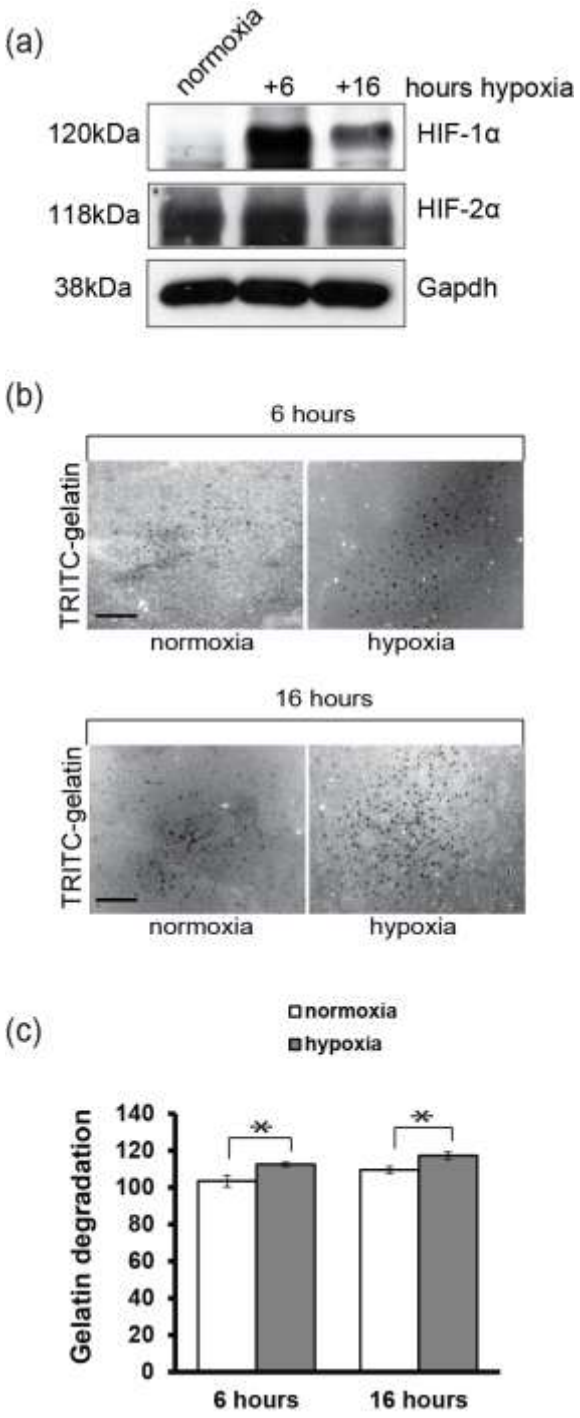

Figure 4.9 MDA-MB-231 cells incubated under hypoxic conditions increase gelatin degradation. **(a)** MDA-MB-231 cells were seeded at $3X10^4$ cells/ml in a 6-well plate, allowed to adhere and incubated in normoxia and hypoxia for 6 and 16 hours. Cells were then lysed for 10 minutes in lysis buffer (see materials and methods). Equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of HIF-1α (Cell Signaling Technology). Membrane was stripped and re-probed for levels of HIF-2α (Santa Cruz). Gapdh, a house-keeping protein was used as a loading control. **(b)** Cells were seeded on gelatin-coated coverslips and incubated in normoxia and hypoxia for 6 and 16 hours. **(c)** The results shown are mean grey value ±SEM of 30 fields of view from each experimental condition over three separate experiments. Statistical significance compared with normoxia was calculated using Student's *t*-test; *, $P<0.05$.

4.3 Discussion

During tumour progression, oxygen tension in the microenvironment surrounding the tumour cells is reduced due to insufficient delivery of oxygen to the demand of the tissues, resulting in hypoxia (Brown and William, 2004; Fyles et al., 2006; Kimbro and Simons, 2006; Travers, 2006). Cancer cells resist the negative effects of hypoxia predominantly via the activity of transcription factor hypoxia-inducible-factor-1 (HIF-1) (Semenza and Wang, 1992). It is well established that HIF-1α can promote angiogenesis by up-regulating the secretion of VEGF into the tumour stroma, hence maintaining oxygen supply and supporting tumour growth (Sullivan and Graham, 2007). However, tumour hypoxia is linked to increased metastatic potential (Goonewardene et al., 2002; Harris, 2002; Schindl et al., 2002), although the molecular mechanisms involved remain unclear.

In this chapter, the effect of increased HIF-1α expression was tested on the chosen breast carcinoma cell lines MDA-MB-231 and MCF-7. HIF-1α stabilization was successfully induced by using an established protocol to mimic hypoxia with DMOG, which is a cell-permeable, non-toxic prolyl-4-hydroxylase inhibitor which allows HIF-1α to accumulate in cells (Milkiewicz et al., 2004). It is demonstrated here that DMOG treatment significantly increased invadopodia formation in MDA-MB-231 cells **(figure 4.2).** It is surprising to detect an increase of invadopodia formation as early as 6 hours in the presence of DMOG, indeed the increased formation of invadopodia was lost by 16 hours (data not shown). This suggests that HIF-1α is rapidly translocated into the nucleus and that transcriptional modulation of gene expression associated with invasive capacity occurs very quickly. Indeed, others have reported changes in protein levels following as little as 3 hours DMOG treatment (Hudecova et al., 2011). Interestingly, sustaining the presence of DMOG in cells throughout the assay exhibited a further elevated response **(figure 4.2),** thus indicating the higher the level of HIF-1α that is maintained the more invasive the cell population becomes. Not only was increased invadopodia activity detected but also a substantial increase in the rate of gelatin-matrix degradation **(figure 4.2).**

These results suggested that HIF-1α was directly influencing the behaviour of the cancer cells. DMOG inhibits the activity of prolyl and asparaginyl hydroxylases at two proline and one asparaginyl residues (Milkiewicz et al., 2004) but whilst the current data and others have reported that DMOG activity leads to stabilization of HIF-1α expression, there is the possibility of off target effects. Indeed, DMOG activity has been associated with non-HIF-1α-related events (Elvidge et al., 2006). However, specific reduction of HIF-1α expression was able to attenuate the ability of DMOG to enhance increased invadopodia forming potential in the MDA-MB-231 cells, thus providing further support for a specific role for HIF-1α on invadopodia formation. Since the work presented here was initiated it has been reported that hypoxia can influence invadopodia formation in fibrosarcoma cells (Lucien et al., 2011), however this study only evaluated the effects of hypoxia and did not specifically address the role of HIF-1α.

To further explore the role of HIF-1α in invadopodia formation, an overexpression strategy was utilised. This particular expression system was sufficient to overcome endogenous HIF-1α degradation pathways for up to 72 hours and deliver a significant increase in HIF-1α expression. Other studies using WT-HIF-1α constructs incorporated the treatment of MG132, a proteasome degradation inhibitor to stabilize HIF-1α expression (Choi et al., 2011). Hypoxia can also trigger HIF-2α expression and it can interact with HREs to up regulate gene expression (WENGER, 2002). Importantly, over-expression of HIF-1α did not alter the levels of HIF-2α. Consistent with DMOG studies, GFP-HIF-1α overexpressing cells exhibited a substantial increase in both invadopodia and matrix-degrading ability **(figure 4.6).**

To compliment the HIF-1α induction/over-expression studies, a hypoxia incubator was used to test the invasive potential of MDA-MB-231 cells under hypoxic conditions. Normoxic and hypoxic cells were subjected to an invadopodia assay and hypoxic cells demonstrated a significant increase in gelatin-matrix degradation which is consistent with previous experiments **(figure 4.9).** Collectively, these data strongly support a role for hypoxia, via HIF-1α activity in driving invasive potential of breast cancer cells.

It is well established that invadopodia extension into the matrix relies on force driven by actin polymerization (Schoumacher et al., 2010; Wolf and Friedl, 2009) and

matrix-degrading proteases particularly MT1-MMP (Poincloux et al., 2009). The enhancement of invadopodia formation and degradation activity in response to increased expression of HIF-1α may be via regulation of MT1-MMP. HIF-1 is involved in desferoxamine-induced invasion in glioblastoma cells by activating proteolytic enzymes responsible for ECM degradation, such as MMP-2, MMP-9, MT1-MMP and urokinase plasminogen activator receptor (uPAR) (Elstner et al., 2007). Additionally, HIF-1α has been demonstrated to promote invasion of breast cancer cells through a MMP-9 and Ras dependent mechanism (Choi et al., 2011). Moreover, hypoxia has been shown to promote RhoA-dependent trafficking of MT1-MMP to the plasma membrane (Munoz-Najar et al., 2006) hence facilitating ECM degradation.

In parallel with MDA-MB-231 cells, the effects of DMOG treatment and GFP-HIF-1α were tested in MCF-7 cells. Increased HIF-1α expression using both methods resulted in dissociation of cells from their colonies **(figure 4.3)**. This observation was quantified and a significant increase in the percentage of cells attempting to escape from colonies was observed **(figure 4.3 and 4.8)**. Cell shape analyses also revealed that DMOG treatment reduced cell spread area and increased cell elongation **(figure 4.3).** This behaviour is likely to be a pre-requisite of invasion/invadopodia formation, by which once the cells escaped from colonies and become single cells, they could increase spread area and begin to invade as observed in chapter 3 **(figure 3.5).** Others have shown that hypoxia affects cytoskeletal re-organization by increasing stress fibres and membrane ruffling (Huot et al., 1998) but a specific effect of HIF-1α overexpression on MCF-7 has not been reported. It was surprising to detect a reduction of E-cadherin positive cell junctions which was correlated with a significant reduction in expression at a protein level during HIF-1α over-expression **(figure 4.8 a and b)**. Indeed, others have reported changes in E-cadherin expression that was associated with increased HIF-1α expression in ovarian carcinomas (Imai et al., 2003).

E-cadherin expression can be transcriptionally suppressed by other transcription factors such as Snail (Blechschmidt et al., 2008) and HIF-1α has been shown to up-regulate Snail expression (Imai et al., 2003; Luo et al., 2011). Indeed, in renal cell carcinoma dysregulation of HIF-1 activity contributes to the down-regulation of E-

cadherin expression, loss of cell-cell adhesion, and the epithelial-to-mesenchymal transition (EMT) (Krishnamachary et al., 2006).

Similar finding were also reported in gastric cancer cells where HIF-1 up-regulation contributed to the EMT phenotype and increased invasiveness (Zhou et al., 2011). Furthermore, DMOG has also been reported to inhibit LPS-induced TNF-alpha upregulation in macrophages (Takeda et al., 2009).

Data presented here also implies that there is a synergistic relationship between hypoxia and growth factor stimulation where combined addition of DMOG and HGF leads to a significant increase in cell:cell dissociation above DMOG stimulated levels. HGF is known to be a potent inducer of cell: cell dissociation and scattering (Bright et al., 2009; Royal and Park, 1995; Wells et al., 2005), and hypoxia has been shown to increase both the level of c-met and HGF expression (Eckerich et al., 2007; Ide et al., 2006). Moreover, c-met signalling can also lead to increased HIF-1α expression (Tacchini et al., 2001). Thus, it is plausible to speculate that this symbiotic expression relationship contributed to the increase in cell: cell dissociation detected here.

Although increased expression of HIF-1α in MCF-7 cells induced significant effect on cell-cell dissociation, no induction of invadopodia formation was detected. This is in contrast to the robust effect of DMOG/ HIF-1α over-expression on the highly invasive MDA-MB-231 cells. It would appear that in MCF-7 cells, HIF-1α over-expression alone is insufficient to induce invadopodia formation. Indeed, MCF-7 cells also did not respond to EGF or HGF stimulation suggesting that these cells lack intrinsic components of the invasive machinery. It would be interesting to make a comparison of the cytoskeletal and matrix degrading protein expression patterns between these two cell lines.

Work presented in this chapter clearly shows that increased HIF-1α expression by chemical or over-expression strategies can enhance invadopodia formation in invasive breast cancer cells. Moreover, exposure of cells to a hypoxic environment leads to increased levels of invadopodia formation and matrix degradation.

Chapter 5 - PAK1 and PAK2 are required for invadopodia formation in breast cancer cells

Chapter 5 – PAK1 and PAK2 are required for invadopodia formation in breast cancer cells

5.1 Introduction

Chapter 3 and 4 demonstrated that invadopodia formation could be induced by either growth factor stimulation or increased HIF-1α expression. An invasion process requires the production of signalling proteins that control the actin cytoskeleton. Among the proteins known to play a major role in the re-organization of the actin cytoskeleton are p21-activated kinases (PAKs).

5.1.1 PAK1 and PAK2 in breast cancer invasion

Over-expression and/or hyperactivation of PAK1 and PAK2 isoforms have been frequently observed in several human cancers (Kumar et al., 2006). PAK1 is localised to genomic regions, which are frequently amplified in a number of cancer cells including breast cancers. PAK expression is deregulated in breast cancers, and increased PAK1 activity correlates well with the invasiveness of human breast cancer cells and tumours via a correlation between high grades, protein level and kinase activity of PAK1 (Shrestha et al., 2011). PAK2 has also been found to be highly expressed in breast cancer cell lines and highly invasive breast carcinoma tissues (Li et al., 2011). However, little is known about the role of PAK1 and PAK2 in the formation of invadopodia of human breast carcinoma cells with and/or without extracellular stimulations. Previous work using PAK1-AID has suggested that PAK activity is required to sustain invadopodia formation and activity in invasive metastatic human melanoma cell line A375MM (Ayala et al., 2008). This chapter describes the involvement of PAK1 and closely related family member PAK2 in the formation of invadopodia in the chosen MDA-MB-231 and MCF-7 cell lines.

5.2 Results

5.2.1 PAK1 and PAK2 are expressed in different breast cancer cells

Initially, the expression level of PAK1 and PAK2 was assayed in breast cancer cell lines known to have different degrees of invasive ability. The non-tumorigenic MCF-10A cells express low level of PAK1 and PAK2, whereas the highly invasive MDA-MB-231 cells express consistently higher level of PAK1 and PAK2 **(figure 5.1).** This indicates that expression level of PAK1 and PAK2 correlated with the level of invasive ability of these cells. The PAK1 and PAK2 antibodies are isoform specific and do not cross-react with any other PAK family members (Bright et al., 2009).

5.2.2 PAK1 is phosphorylated downstream of HGF in MDA-MB-231 and MCF-7 cells

Work presented here (chapter 3) demonstrated that HGF can stimulate invadopodia forming activity. HGF interaction with the c-Met receptor initiates a number of signalling cascades and it has been shown to specifically activate PAK1 (Bright et al., 2009; Hunter and Zegers, 2010; Royal et al., 2000), but this has not been reported in breast cancer cells. Thus, the activation of PAK1 and closely related family member PAK2 was monitored downstream of HGF in breast cancer cells using an antibody that recognises an autophosphorylation site in both proteins. Several autophosphorylation sites have been identified, including serines 199 and 204 of PAK1 and serines 192 and 197 for PAK2 (Manser et al., 1997). In this study, phospho-PAK1/PAK2 antibody detects endogenous PAK1 and PAK2 when phosphorylated at Thr423 and Thr402 respectively. A 60-minute time-course experiment was performed on cells seeded on either plastic or gelatin matrix, followed by Western blot analysis. For cells seeded on plastic, PAK1 became phosphorylated on Thr423 within 5 minutes of HGF stimulation in MCF-7 **(figure 5.2)** and after 15 minutes in MDA-MB-231 cells **(figure 5.2)**. The phosphorylation in MDA-MB-231 cells was maintained for up to 60 minutes **(figure 5.2)**, whereas in MCF-7 PAK1 phosphorylation peaked at 15 minutes **(figure 5.2).** Cells seeded on gelatin demonstrated a slightly different trend in which consistently higher PAK1 phosphorylation downstream of HGF was observed. In MDA-MB-231 cells, PAK1 phosphorylation also increased **(figure 5.3c and d)** but not as robustly as in MCF-7 cells (**figure 5.3c and d).**

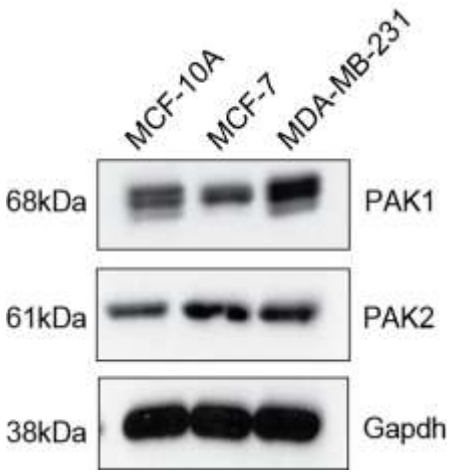

Figure 5.1 PAK1 and PAK2 are expressed in MCF-10A, MCF-7 and MDA-MB-231 cells. Whole cell lysates of growing cells were immunoblotted for PAK1 and PAK2 expression. Gapdh, a house-keeping protein is used as loading control. These results are representative of three independent experiments.

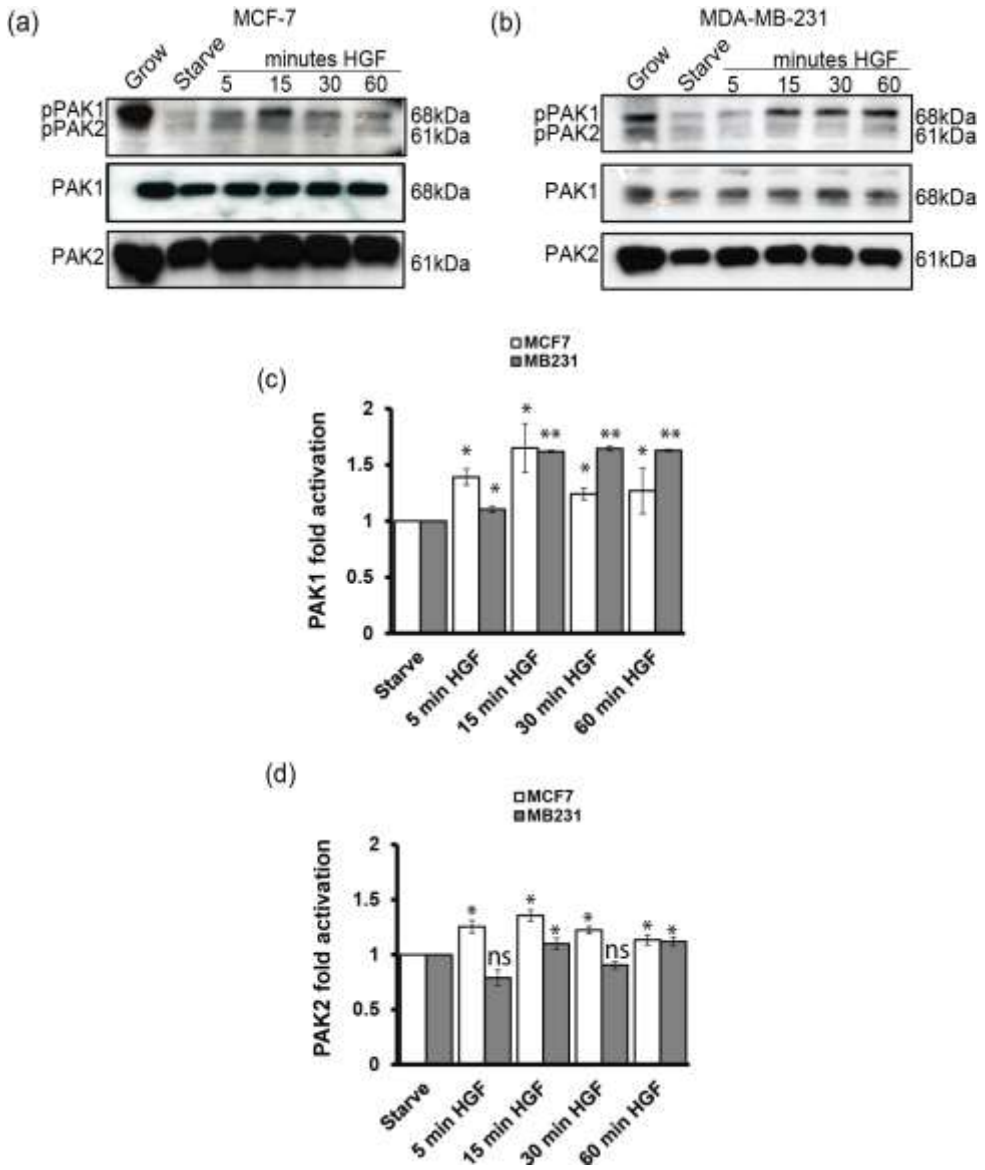

Figure 5.2 PAK1 and PAK2 are phosphorylated by HGF in MCF-7 and MDA-MB-231 cells seeded on plastic. (a and b) Cells were seeded at $3X10^4$/ml and allowed to adhere for 24 hours. Cells were serum-starved for 24 hours and stimulated with HGF for up to 60 minutes. Cells were lysed for 10 minutes in lysis buffer (see materials and methods). Equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of phospho-PAK1 and PAK2 (Thr423/Thr402) (Cell Signaling Technology). **(c and d)** Densitometric analysis was performed to measure the phospho-PAK1 and PAK2 signals that were normalised to total level of PAK1 and PAK2 respectively. These results are representative of three independent experiments. Statistical significance compared with starve was calculated using Student's *t*-test; *, $P<0.05$. **, $P<0.005$, ns=non-significant

5.2.3 PAK2 is phosphorylated downstream of HGF in MDA-MB-231 and MCF-7 cells

For cells seeded on plastic, PAK2 became phosphorylated on Thr402 within 5 minutes of HGF stimulation in MCF-7 **(figure 5.2a)** but inconsistent phosphorylation was observed in MDA-MB-231 cells in which PAK2 phosphorylation was difficult to detect **(figure 5.2b).** In contrast, cells seeded on gelatin revealed a more pronounced effect; that PAK2 phosphorylation in MCF-7 was seen as early as 5 minutes post-HGF stimulation **(figure 5.3a).** Whereas in MDA-MB-231 cells, PAK2 phosphorylation was increased even in starved cells, followed by a further increase at 5 minutes HGF stimulation and maintained for up to 60 minutes **(figure 5.3b).**

5.2.4 Efficient knockdown of PAK1 and PAK2 protein expressions

PAK1 and PAK2 are both responsive to HGF when seeded on gelatin matrix **(Figure 5.3)** and HGF can stimulate invadopodia formation **(chapter 3)**. Thus the requirement for PAK1/PAK2 during invadopodia formation was determined. shRNA and siRNA smartpool oligos were tested for specific knockdown of PAK1 and PAK2. Both strategies effectively knocked down expression of PAK1/2 in MDA-MB-231 cells**.** Knockdown of one PAK isoform did not affect the expression level of the other **(figure 5.4a and b)**.

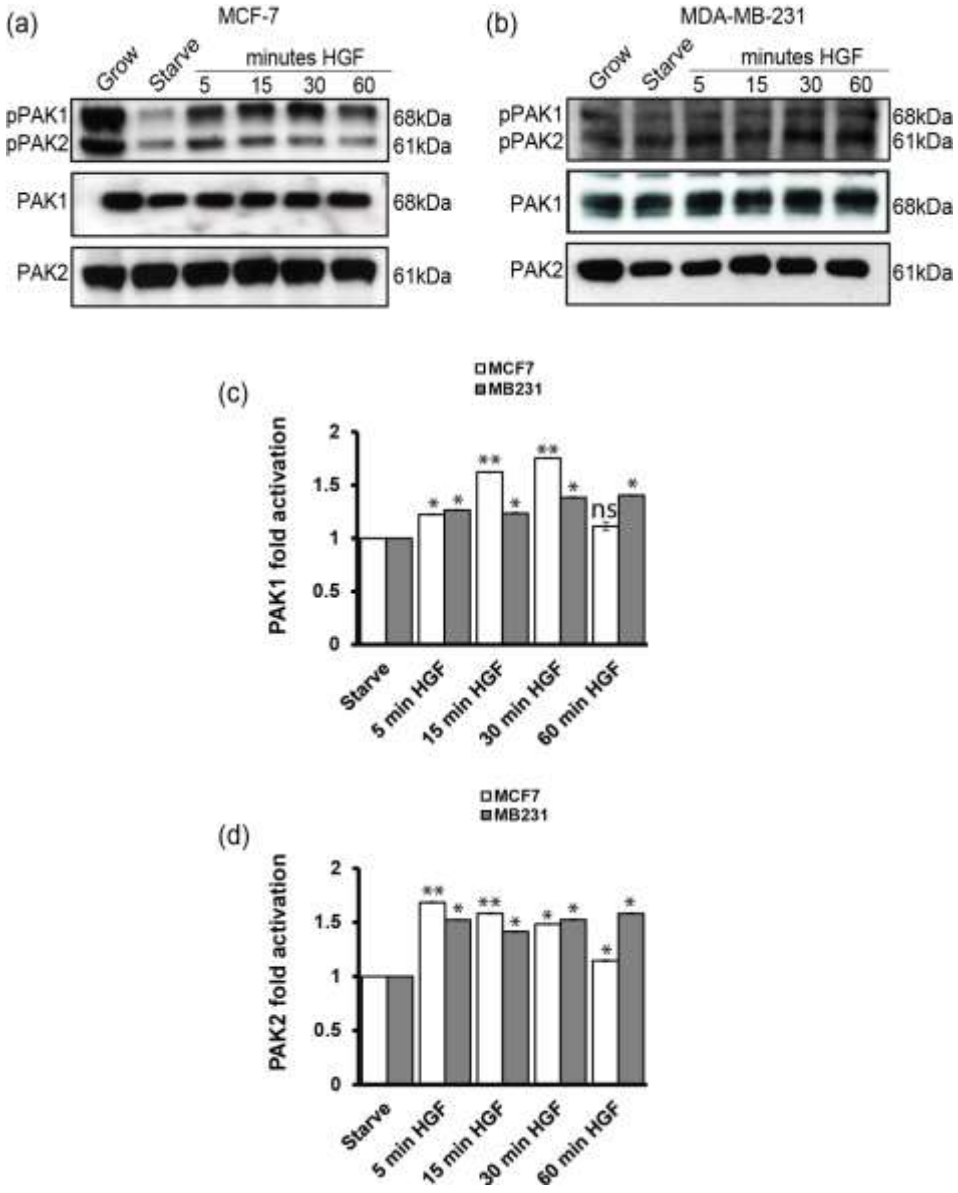

Figure 5.3 PAK1 and PAK2 are phosphorylated by HGF in MCF-7 and MDA-MB-231 cells seeded on gelatin matrix. (a and b) 6-well plates were coated with gelatin prior to seeding cells. Cells were seeded at $3X10^4$/ml and allowed to adhere for 24 hours. Cells were serum-starved for 24 hours and stimulated with HGF for up to 60 minutes. Cells were lysed for 10 minutes in lysis buffer (see materials and methods). Equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of phospho-PAK1 and PAK2 (Thr423/Thr402) (Cell Signaling Technology). **(c and d)** Densitometric analysis was performed to measure the phospho – PAK1 and PAK2 signals that were normalised to total level of PAK1 and PAK2 respectively. These results are representative of three independent experiments. Statistical significance compared with starve was calculated using Student's *t*-test; *, $P<0.05$. **, $P<0.005$, ns=non-significant

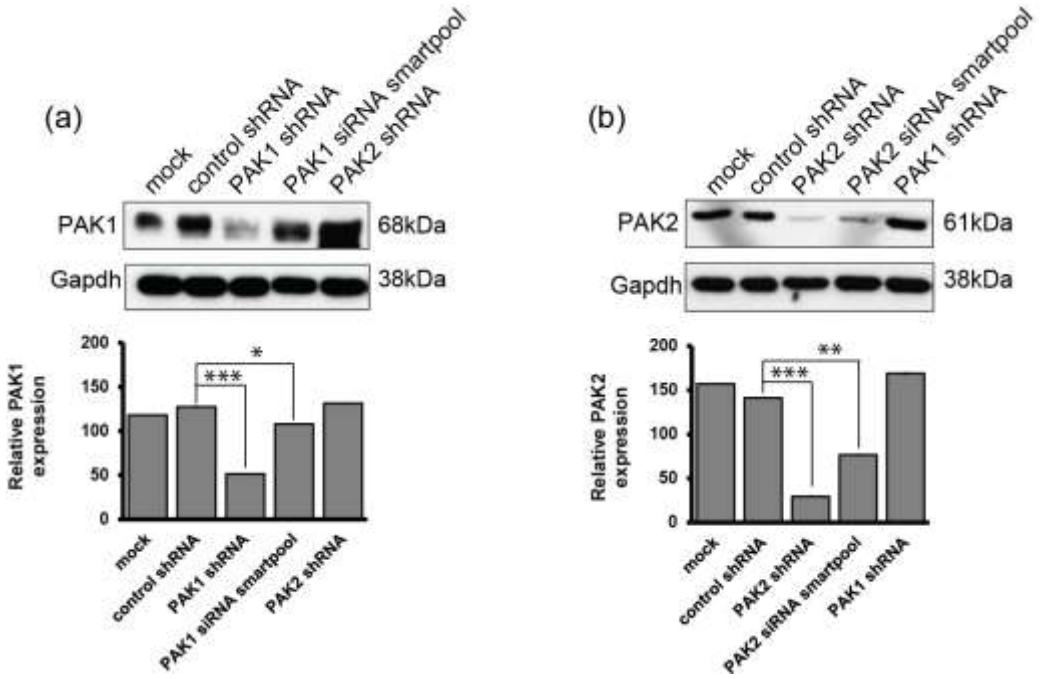

Figure 5.4 PAK1 and PAK2 expression are successfully reduced in MDA-MB-231 cells. (a and b) MDA-MB-231 cells were seeded at $1X10^5$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with mock, control shRNA, PAK1 shRNA and PAK2 shRNA oligos for 24 hours. Puromycin was added to the transfected cells for 48 hours post-transfection. PAK1 siRNA smartpool was transfected in cells every 24 hours for 48 hours. Cells were lysed for 10 minutes in lysis buffer (see materials and methods). Equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of PAK1 and PAK2 (Cell Signaling Technology). Gapdh, a house-keeping protein was used as a loading control. Relative expression of PAK1 and PAK2 was measured using densitometric analysis. The results shown are representative of three separate experiments. Statistical significance compared with control shRNA was calculated using Student's *t*-test; *, $P<0.05$. **, $P<0.005$. ***, $P<0.0005$.

5.2.5 PAK1 is required for invadopodia formation

The shRNA vector is bi-cistronic whereby shRNA expressing cells also express GFP to aid visualisation and quantification processes. Invadopodia formation in control shRNA-transfected cells is consistent with the percentage observed under basal condition as shown in Chapter 3 **(figure 5.5b).** However, PAK1 shRNA markedly reduced invadopodia formation in MDA-MB-231 cells **(figure 5.5a and b).** To determine that this effect was specifically due to the loss of PAK1 expression, a rescue experiment was performed. Technical difficulties prevented the generation of an RNAi-resistant PAK1 mutant, thus wild-type mouse PAK1 was used. Wild-type mouse PAK1 successfully rescued the phenotype observed in PAK1 knockdown cells whereby the percentage of cells forming invadopodia increased to almost control level **(figure 5.5a and b).** In parallel, wild type human PAK2 was also used in the rescue experiment. However, PAK2 over-expression was not able to rescue the PAK1 knockdown phenotype.

5.2.6 PAK2 is required for invadopodia formation

A reduction in PAK1 expression significantly reduced invadopodia formation and this phenotype could not be rescued by the presence of endogenous or overexpressed PAK2. Thus it might be expected that PAK2 is not involved in invadopodia formation. However, PAK2 shRNA also markedly reduced invadopodia formation in MDA-MB-231 cells compared to control **(figure 5.6a and b).** To determine whether this effect is specifically due to the loss of PAK2 expression a rescue experiment was performed. A wild type PAK2-resistant mutant was generated and used in the rescue experiment. WT-PAK2^{r} successfully rescued the phenotype observed in PAK2 knockdown cells in which the percentage of cells forming invadopodia increased to almost control level **(figure 5.6a and b).** In parallel, wild type mouse PAK1 was tested and exhibited no rescue phenotype **(figure 5.6a and b).**

5.2.7 HGF and DMOG could not induce invadopodia in PAK1/2 depleted cells

PAK1 and PAK2 knockdown significantly decrease invadopodia formation suggesting an essential requirement for these proteins. It might be speculated therefore that PAK1 and/or PAK2 would be required for the increased invadopodia

activity demonstrated in the presence of DMOG. Thus, the effect of DMOG treatment in the background of PAK1/2 depletion was examined. As anticipated, in a background of PAK1/2 knockdown, DMOG treatment was unable to induce invadopodia formation in cells to control levels. However, there was still an induction of invadopodia formation in the PAK1/2 knockdown background **(figure 5.7a and b),** suggesting that DMOG works through multiple signalling pathways to induce invadopodia formation**.**

In parallel with DMOG, the effect of adding HGF to the PAK1/PAK2 depleted cells was also examined. Similar to DMOG, HGF stimulation was unable to induce invadopodia formation in PAK1/PAK2 knockdown cells to control levels **(figure 5.8).** Indeed, induction of invadopodia formation in PAK1/PAK2 depleted cells was significantly reduced, suggesting these proteins are required during HGF-stimulated invadopodia formation.

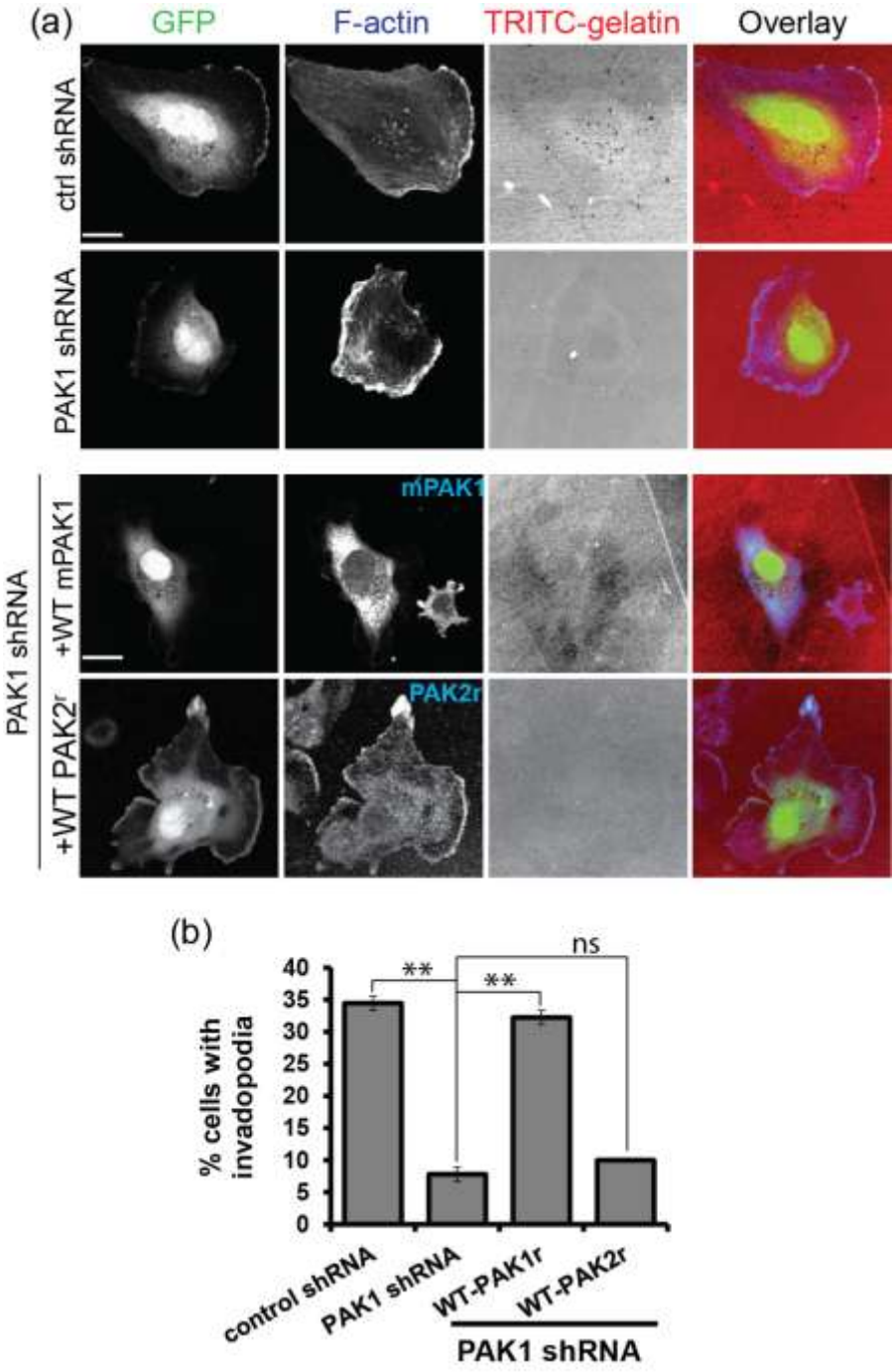

Figure 5.5 The loss of PAK1 expression reduces invadopodia formation in MDA-MB-231 cells. (a) MDA-MB-231cells were seeded at $1X10^5$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with mock, control shRNA and PAK1 shRNA for 48 hours. Puromycin was added to the transfected cells for 48 hours post-transfection for transient selection. At 48 hours after transfection, WT-mouse-$PAK1^r$ and WT-human-$PAK2^r$ were transfected into the knockdown cells for 24 hours. Cells were seeded on gelatin-coated coverslips for 3 hours followed by fixation with 4% PFA. Control and PAK1 shRNA cells were stained for F-actin using Phalloidin 633. **(b)** Cells were scored for the presence of actin puncta that colocalize with area of degradation on the gelatin and F-actin (in control and PAK1 shRNA) and for the co-expression of PAK1 shRNA and rescue-mutant (for rescue experiments). The mean percentage of cells with invadopodia formation was calculated. Scale bar=10µm. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with control shRNA was calculated using Student's *t*-test; **, P<0.005, ns=non-significant.

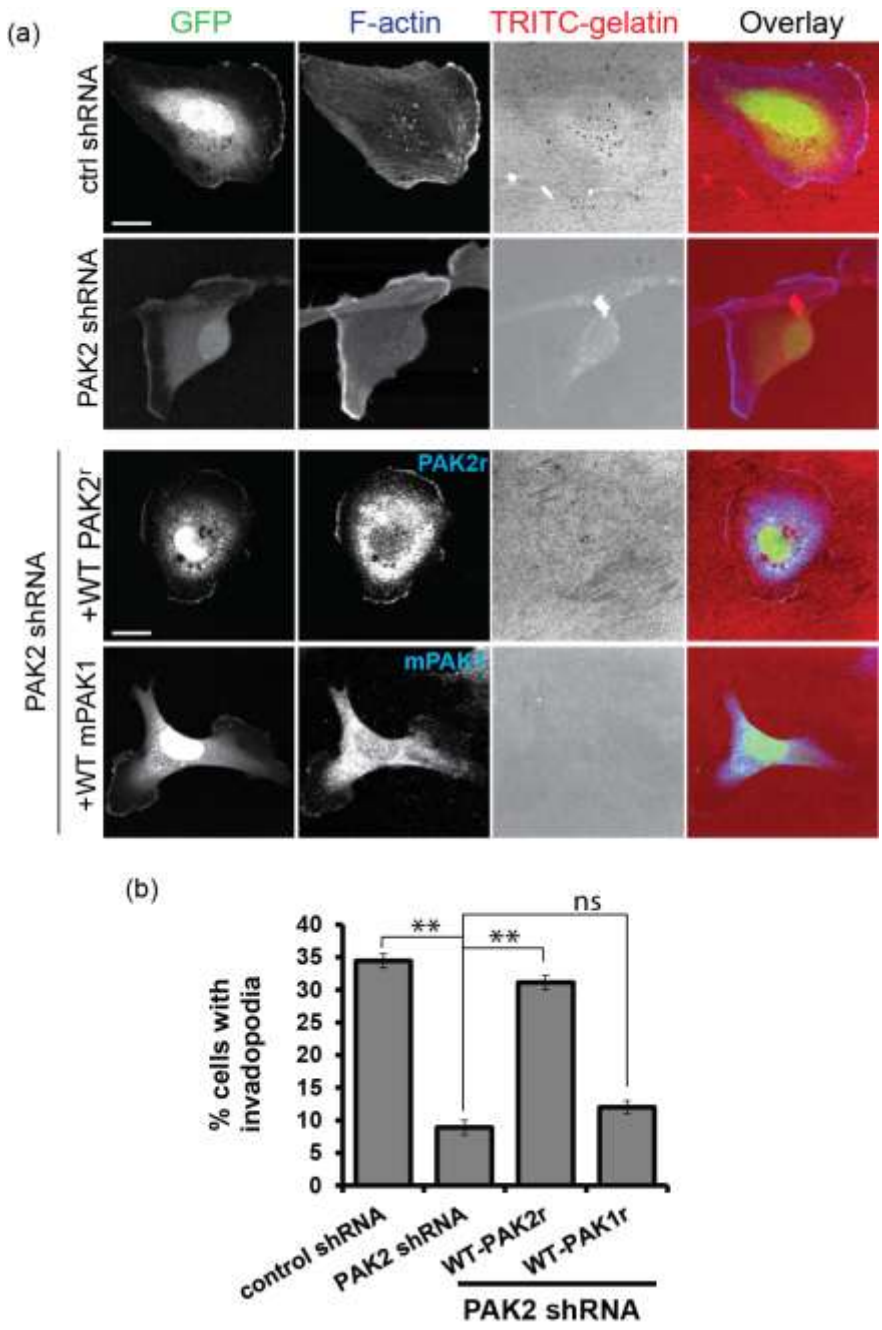

Figure 5.6 The loss of PAK2 expression reduces invadopodia formation in MDA-MB-231 cells (a) MDA-MB-231cells were seeded at $1X10^5$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with mock, control shRNA, and PAK2 shRNA oligos for 48 hours. Puromycin was added to the transfected cells for 48 hours post-transfection for transient selection. At 48 hours after transfection, WT-mouse-PAK1^r and WT-human-PAK2^r were transfected into the knockdown cells for 24 hours. Cells were seeded on gelatin-coated coverslips for 3 hours followed by fixation with 4% PFA. Control and PAK1 shRNA cells were stained for F-actin using Phalloidin 633. **(b)** Cells were scored for the presence of actin puncta that colocalize with area of degradation on the gelatin and F-actin (in control and PAK2 shRNA) and for the co-expression of PAK2shRNA and rescue-mutant (for rescue experiments). The mean percentage of cells with invadopodia formation was calculated. Scale bar=10µm. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with control shRNA was calculated using Student's *t*-test; **, P<0.005. ns=non-significant.

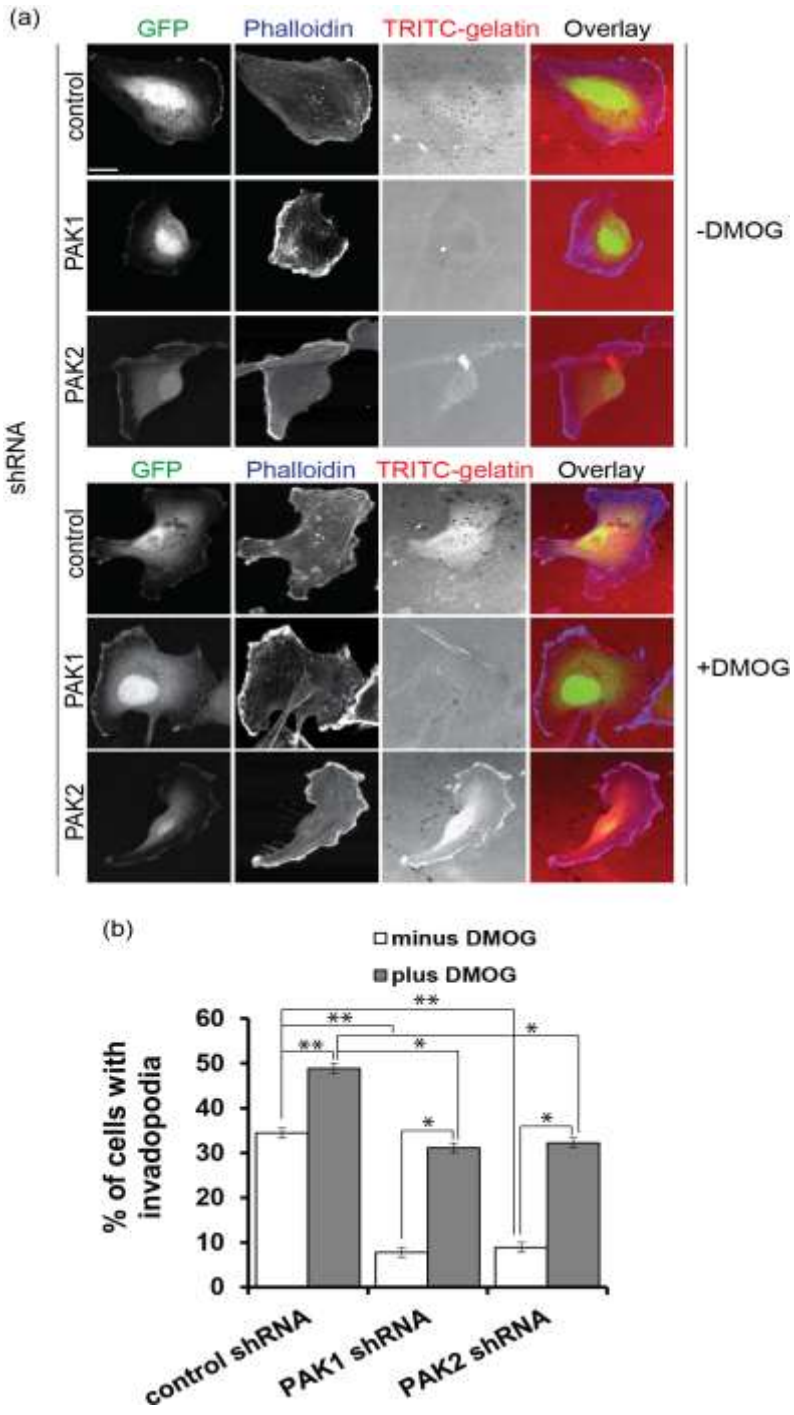

Figure 5.7 DMOG cannot fully rescue invadopodia defect in PAK1/PAK2 depleted MDA-MB-231 cells. Cells were seeded at $1X10^5$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with control shRNA and PAK1 shRNA for 48 hours. Puromycin was added to the transfected cells for 48 hours post-transfection for transient selection. At 48 hours after transfection, cells were re-seeded on gelatin-coated coverslips for 3 hours in the presence or absence of DMOG followed by fixation with 4% PFA. Cells were stained for F-actin using Phalloidin-633. **(b)** Cells were scored for the presence of F-actin puncta that colocalize with area of degradation on the gelatin. The mean percentage of cells with invadopodia formation was calculated. Scale bar=10µm. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with control shRNA or control shRNA +DMOG was calculated using Student's *t*-test; *, P<0.05. **, P<0.005.

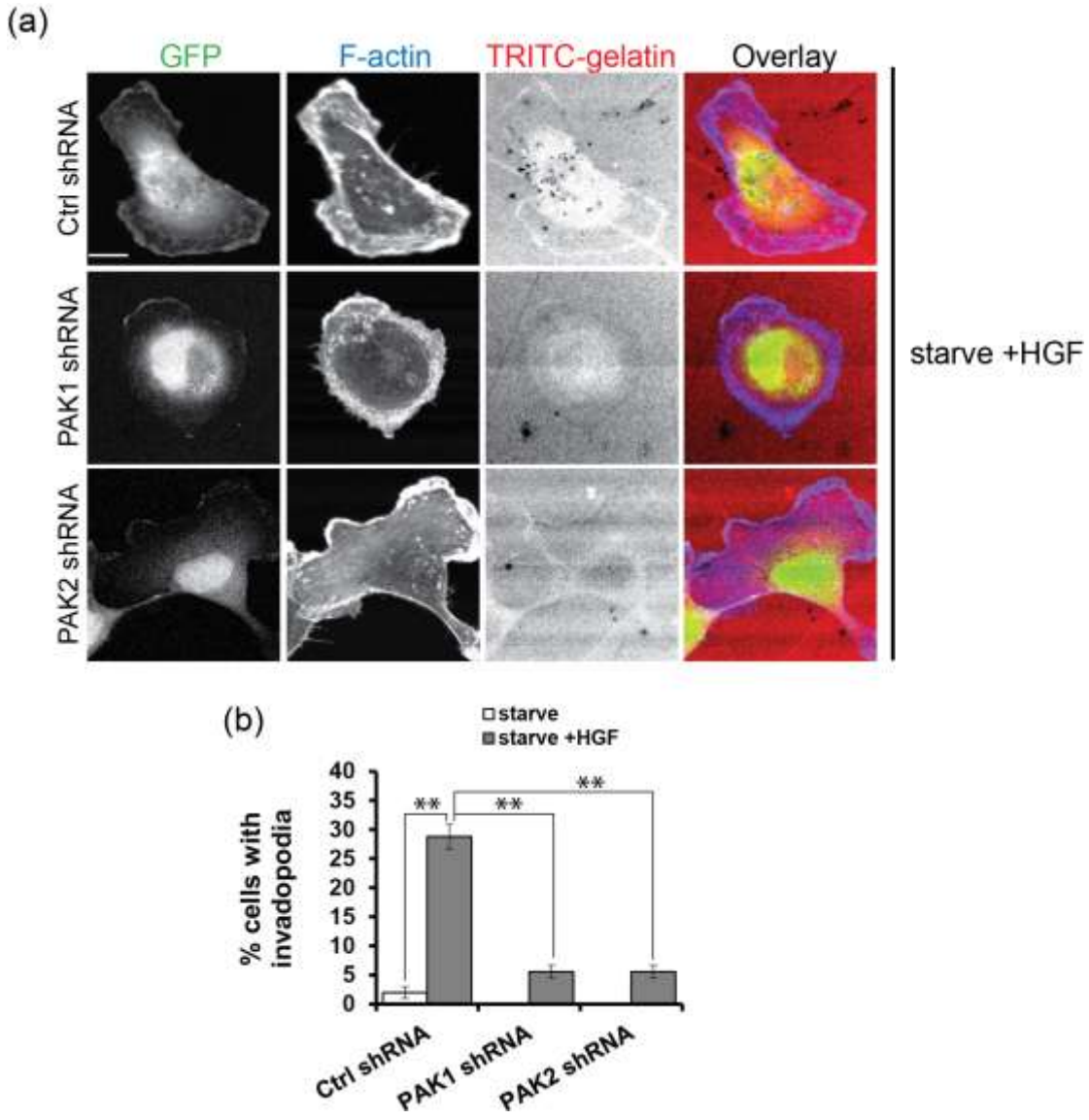

Figure 5.8 HGF could not induce invadopodia formation in the PAK1/2 knockdown MDA-MB-231 cells. Cells were seeded at $1X10^5$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with control shRNA and PAK1 shRNA for 48 hours. Puromycin was added to the transfected cells for 48 hours post-transfection for transient selection. At 48 hours after transfection, cells were starved for at least 16 hours before being re-seeded on gelatin-coated coverslips for 3 hours in the presence or absence of HGF followed by fixation with 4% PFA. Cells were stained for F-actin using Phalloidin-633. **(b)** Cells were scored for the presence of F-actin puncta that colocalize with area of degradation on the gelatin. The mean percentage of cells with invadopodia formation was calculated. Scale bar=10µm. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with control shRNA or control shRNA +HGF was calculated using Student's *t*-test; **, P<0.005.

5.2.8 PAK1 and PAK2 are localised at invadopodia

PAK1 and PAK2 are both required for invadopodia formation **(figure 5.5 and 5.6)** but cannot functionally compensate for each other. To further explore the specific role of these two proteins, their subcellular localisation was examined. Wild type PAK1 and PAK2 were cloned into a GFP-tagged expression vector using Gateway Cloning Technology to generate GFP-PAK1 and GFP-PAK2. PAK1 and PAK2 over-expressing cells or control cells expressing GFP alone were seeded on gelatin-coated coverslips and stained for F-actin. As expected, there was no co-localisation between GFP and F-actin puncta **(figure 5.9a).** Interestingly, F-actin puncta colocalized with both GFP-PAK1 and GFP-PAK2 at sites of gelatin degradation, thus suggesting that PAK1 and PAK2 are localised at invadopodia **(figure 5.9b and c).**

5.2.9 PAK1 and PAK2 over-expression does not increase invadopodia formation

Both HGF stimulation and DMOG treatment can induce increased invadopodia activity in MDA-MB-231 cells **(Chapter 3)**. Given that PAK1 and PAK2 are localised to invadopodia and required for invadopodia formation, the effect of overexpressing PAK1 and PAK2 on invadopodia formation was examined. Surprisingly, over-expression alone does not affect invadopodia formation in which the percentage of invadopodia remained at approximately 35%, the same level observed in the control **(figures 5.10a and b).**

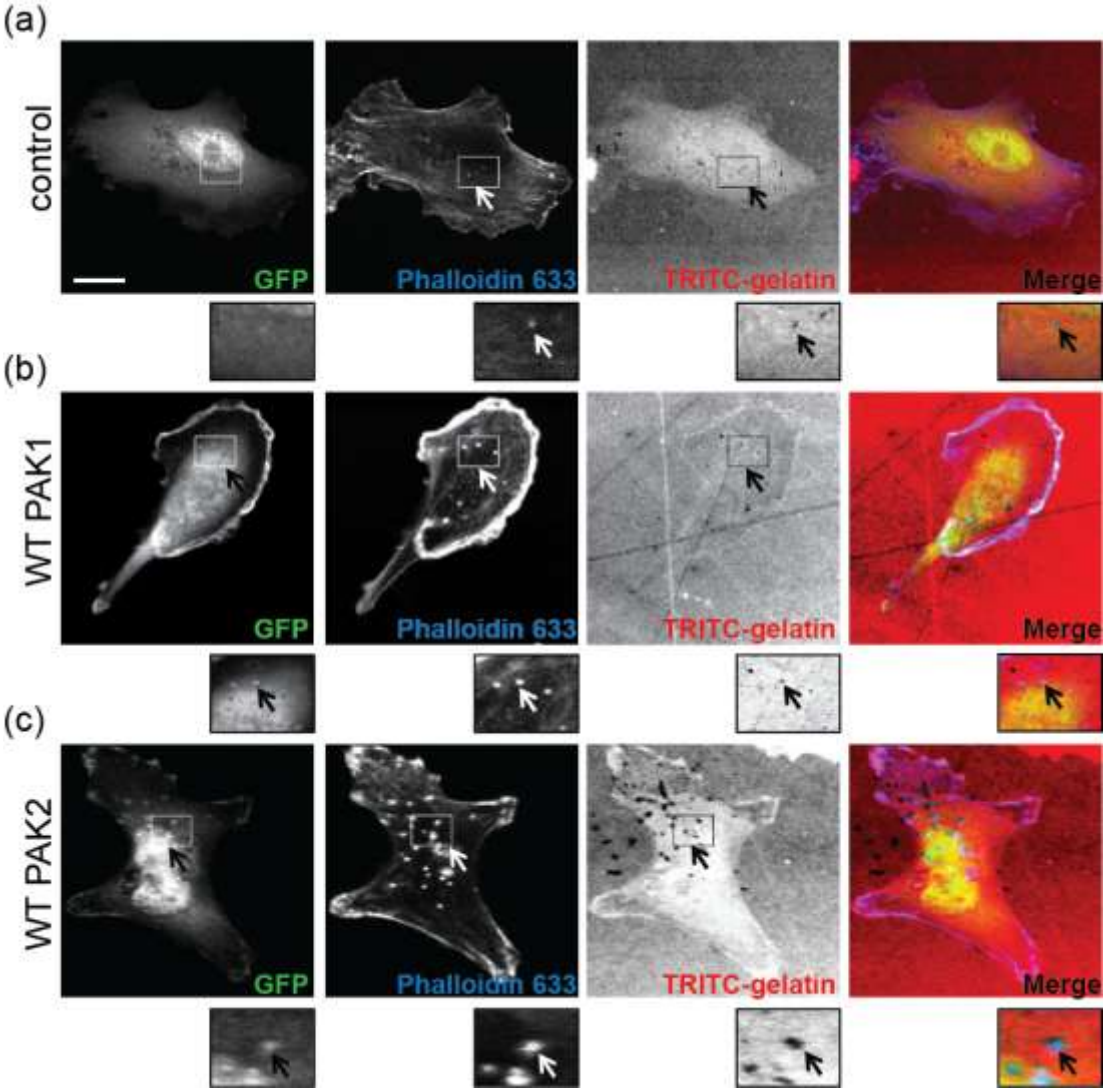

Figure 5.9 PAK1 and PAK2 are localised at invadopodia in MDA-MB-231 cells. Cells were seeded at $1X10^5$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with **(a)** GFP-control, **(b)** WT-GFP-PAK1 and **(c)** WT-GFP-PAK2 for 24 hours. At 24 hours after transfection cells were seeded on gelatin-coated coverslips for 3 hours followed by fixation with 4% PFA. Cells were stained for F-actin using Phalloidin 633. Localisation was observed using confocal microscopy for the presence of F-actin puncta that colocalize with area of degradation on the gelatin and PAK1 and PAK2 over-expression on the GFP channel. Small boxes underneath the cell images are zoomed areas of localisation.

5.2.10 PAK1 and PAK2 over-expression does not induce invadopodia formation in MCF-7 cells

Whilst neither PAK1 nor PAK2 overexpression increased the invadopodia forming activity of MDA-MB-231 cells, over-expression might be able to induce MCF-7 cells to form invadopodia. However, no induction of invadopodia was observed following over-expression of either PAK1 or PAK2 **(figure 5.10b).** PAK1 has been implicated in promoting cell spreading in MDCK cells (Royal et al., 2000), thus cell shape analysis was performed to evaluate whether PAK1/2 contribute to this effect in MCF-7 cells. However, WT-PAK1/2 over-expression alone does not affect cell spread area or cell elongation ratio in these cells **(figure 5.10c and d).**

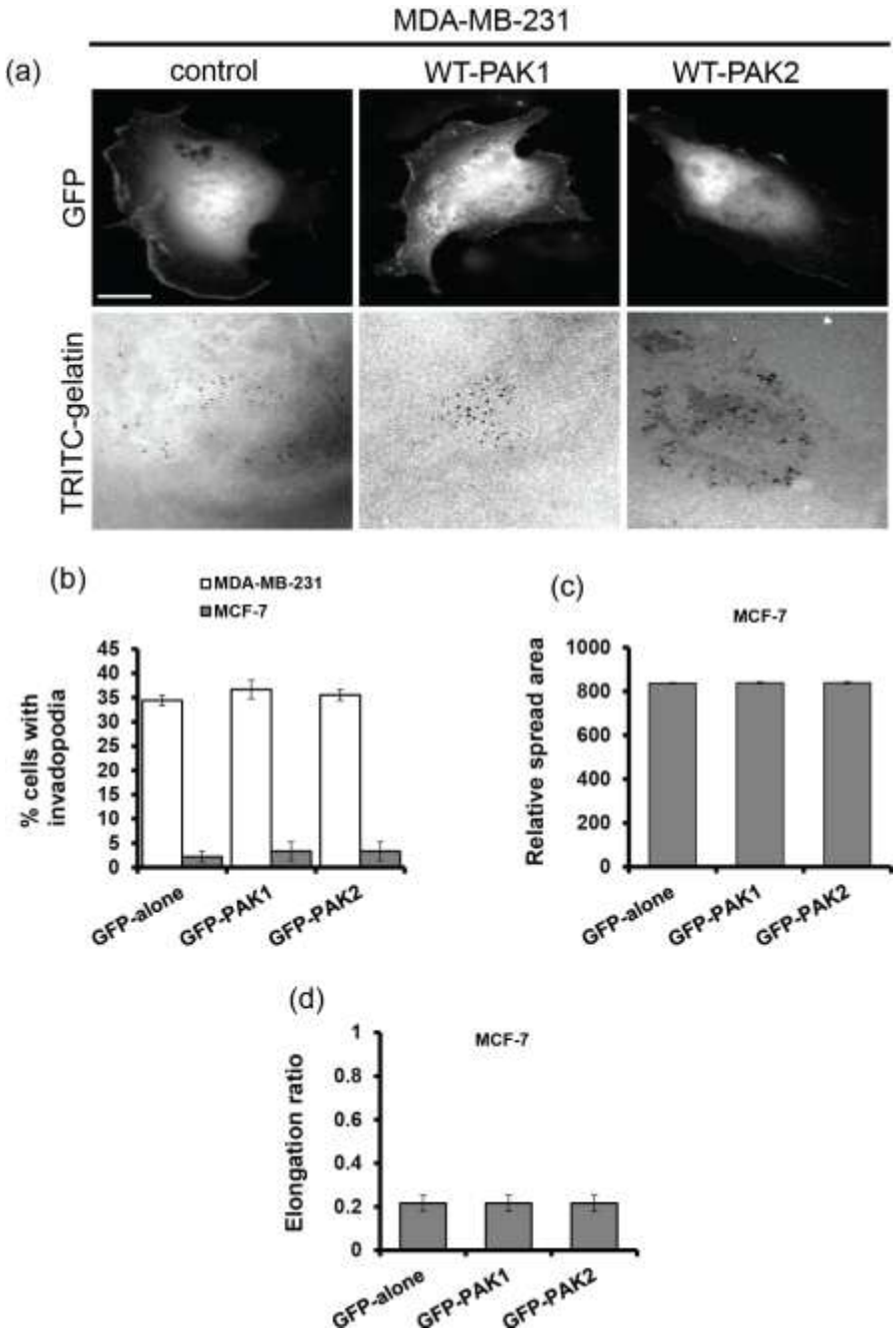

Figure 5.10 PAK1 and PAK2 over-expression does not increase invadopodia formation in MDA-MB231 and MCF-7 cells. (a) MDA-MB-231 and MCF-7 cells were seeded at $1X10^5$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with GFP-control and WT-GFP-PAK1/2 for 24 hours. Cells were seeded on gelatin-coated coverslips for 3 hours, followed by fixation with 4% PFA. **(b)** The mean percentage of cells with invadopodia formation was calculated. Scale bar=10µm. The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments.**(c and d)** The mean relative spread area and elongation ratio of MCF-7 cells over-expressing PAK1/PAK2 was calculated using ImageJ software. Statistical significance compared with GFP-control was calculated using Student's *t*-test.

5.3 Discussion

One of the aims of this study was to identify key proteins that are involved in the invadopodia response downstream of HGF and hypoxia. Invadopodia formation requires remodelling of the actin cytoskeleton; a process known to be regulated by the Rho family small GTP-binding proteins, consisting of the Rho, Rac and Cdc42 subfamilies. p21-activated kinases 1 and 2 (PAK1, PAK2) are major effectors of the Rho GTPases Rac and Cdc42 (Bokoch, 2003). There is some evidence to suggest that PAK1 at least is involved in regulating invadopodia formation (Ayala et al., 2008), but the specific role of PAK1 and PAK2 during the formation of invadopodia in MDA-MB-231 breast carcinoma had not previously been investigated. Moreover, the expression of a kinase-dead PAK1 mutant has implicated PAK1 activity in podosome (actin-rich structures that remodel ECM in normal cells) formation of vascular smooth muscle cells (Furmaniak-Kazmierczak et al., 2007). Podosome formation does not require PAK1 kinase activity; however, catalytic activity regulates the dynamics of podosomes (Webb et al., 2005). In contrast, the involvement of PAK2 on invadopodia formation and matrix degradation has not been studied.

PAK1 and members of the PAK-interacting exchange factor (PIX) family are recent additions to the group of podosome-type adhesion regulatory proteins. In smooth muscle cells, over-expression of PAK1 kinase mutants induces podosome formation, and interaction with β-PIX is necessary to localise PAK1 to the podosomal ring structure (Webb et al., 2005). Similarly, PAK4 localises to macrophage podosomes, and PAK4 kinase activity regulates podosome size and number (Gringel et al., 2006), which is partly dependent on α-PIX. It is likely that a complex consisting of PAK and its regulators binds to the ring structure of podosomes. PAK and PIX could thus form an interface between adhesion-associated proteins of the ring and the regulation of actin dynamics in the core. PAK might regulate F-actin levels by influencing cofilin activity (Gringel et al., 2006) or by phosphorylation of cortactin (Webb et al., 2006).

Both EGF and HGF can induce invadopodia formation **(chapter 3)** (DesMarais et al., 2009; Hwang et al., 2011; Rajadurai et al., 2012; Tague et al., 2004) and PAK1 is known to be activated by these growth factors (Adam et al., 1998; He et al., 2001;

Royal et al., 2000) in a number of cell types including MDA-MB-231 cells (Yang et al., 2011). HGF also activates PAK1 and PAK4 in prostate cancer cells (Bright et al., 2009; Wells et al., 2010) and PAK1 in ovarian cancer cells (Siu et al., 2009). Indeed, it is demonstrated here that PAK1 and PAK2 are activated by HGF at Thr423/Thr402 in MDA-MB-231 and MCF-7 cells **(figure 5.3)** plated on a gelatin matrix. Thus it might be speculated that PAK1 at least acts downstream of HGF to regulate invadopodia formation and RNAi experiments confirmed that PAK1 and PAK2 are required for invadopodia formation in MDA-MB-231 cells **(figure 5.5 and 5.6).** Moreover, the specificity of the RNAi treatment was verified by rescue experiments. This is the first report identifying specific requirements for PAK1 and PAK2 expression during invadopodia formation, and the first time that PAK2 has been linked to invadopodia.

Interestingly, expression of WT-mouse-PAK1 in PAK1 knockdown cells and WT-PAK2^{r} in PAK2 knockdown cells restored the ability to form invadopodia and degrade gelatin matrix **(figure 5.5 and 5.6)**; complimentary expression did not rescue the reduction in invadopodia formation **(figure 5.5 and 5.6).** This strongly suggests that both proteins function during invadopodia formation but their roles are non-overlapping.

Although PAK1 and PAK2 are closely related family members, there are examples of non-overlapping functions in some cells. PAK1 and PAK2 play non-redundant roles in cell–cell junctions/interactions, lamellipodium extension and cell scattering (Bright et al., 2009). PAK1 and PAK2 differentially modulate focal adhesions, by playing opposite roles in regulating the phosphorylation of the myosin light chain (MLC), and RhoA signalling (Coniglio et al., 2008).

The PAK1/2 signalling mechanisms involved in promoting invadopodia are still unclear. However, PAK1 is thought to function along with other proteins such as cortactin to initiate invadopodia formation (Ayala et al., 2008). This interaction may lead to assembly of invadopodia structures and proteases that facilitate ECM degradation such as MTI-MMPs. The PAK1 gene maps to the 11q13 amplicon, the same region that encodes cortactin (Bekri et al., 1997). The main PAK phosphorylation site on cortactin has been shown to be the Ser113 in the first actin-binding repeat (Webb et al., 2006). As a consequence, cortactin binding to F-actin

was reduced suggesting a role for PAK-dependent phosphorylation of cortactin in the regulation of branched actin filament dynamics (Vidal et al., 2002; Webb et al., 2006). There are no previous reports linking PAK2 activity to invadopodia formation; however both PAK1 and PAK2 have been shown to bind to cortactin in resting platelets. After thrombin stimulation, PAK and cortactin dissociate (Vidal et al., 2002), therefore possibly allowing phosphorylated cortactin to trigger remodelling of the actin cytoskeleton. In addition to differences in the cellular behaviour, PAK1 and PAK2 also exhibit differences in the regulation of their kinase activity.

Regulation of PAK1 activity is a complex process involving protein-protein interactions, phosphorylation/dephosphorylation and sphingolipid binding (Bokoch et al., 1998; Chong et al., 2001). PAK1/2 are major effectors of the small GTPases Rac and Cdc42 (Arias-Romero and Chernoff, 2008) and HGF-induced PAK1/2 activation can be attributed to Rac1 and Cdc42 (Bright et al., 2009). The binding of Rac/Cdc42 to the PAK1 regulatory domain induces the phosphorylation of important sites throughout the protein, both by PAK1 itself (Chong et al., 2001) and/or by exogenous kinases such as JAK2, PDK1 and PKA (Howe and Juliano, 2000; King et al., 2000; Rider et al., 2007). PAK2 is unique among the PAK isoforms because it can also be activated through proteolytic cleavage by caspases or caspase-like proteases. Activation of full length PAK2 stimulates cell survival, whereas proteolytic activation of PAK2p34 is involved in apoptosis. PAK2p34 exerts its pro-apoptotic effects via the activation of JNK (Chan et al., 2007; Huang et al., 2009).

Both PAK1 and PAK2 were localised to invadopodia **(figure 5.9).** PAK proteins have been shown to localize to focal adhesions via a PIX/GIT/PKL/paxillin complex (Brown et al., 2002; Manabe et al., 2002; Zhao et al., 2000), but the precise role of PAKs in focal adhesion generation and maturation are still unclear. More recently, focal adhesions have been shown to be able to degrade matrix substrates (Wang and McNiven, 2012). PAK1-induced actin columns in vascular smooth muscle cells containing actin-binding proteins known to be present in podosomes, such as cortactin, the Arp2/3 complex, α-actinin and caldesmon (Kaverina et al., 2003; Tanaka et al., 1993). Therefore, PAK1 and may be PAK2 could be interacting with

these actin-binding proteins to promote invadopodia assembly and facilitate digestion of ECM.

A tight relationship between trafficking and invadopodia formation has been suggested (Baldassarre et al., 2006; Buccione et al., 2009). Staining of PAK1 with a specific antibody in fibroblasts revealed subcellular localization of endogenous PAK1 in sub membranous vesicles (Dharmawardhane et al., 1997). Vesicles are known for their function in trafficking of membrane proteins such as MT1-MMP, which is involved in ECM degradation. Therefore this could in part suggest that PAK1 mediates vesicular trafficking associated with invadopodia formation. In A7r5 cells, the ability of PAK to induce podosomes depends on its interaction with the guanine nucleotide exchange factor PIX (Webb et al., 2005). Similarly, a kinase-dead mutant of PAK1, in which the PIX binding site was eliminated exhibited a severely reduced ability to induce the formation of ECM-degrading invadopodia in VSMCs (Furmaniak-Kazmierczak et al., 2007).

PAK1/2 over-expression alone did not increase invadopodia forming potential of MDA-MB-231 cells **(figure 5.10**) and could not induce MCF-7 cells to form invadopodia or degrade ECM **(figure 5.10).** This is in agreement with similar findings found in other cell types where over-expression of wild-type PAK1 had no significant effect on the overall morphology of the actin cytoskeleton (Frost et al., 1998; Kiosses et al., 1999; Manser et al., 1997; Sells et al., 1997; Webb et al., 2005; Zhao et al., 1998). The lack of effect of wild-type PAK1 on the actin cytoskeleton suggests that PAK activity maybe tightly controlled by regulatory factors such as GTPases, and the cells are able to adjust and maintain optimal PAK activity in spite of over-expression of the wild-type protein. Apart from these studies, other reports have suggested that over-expression of WT-PAK4 alone was unable to elicit any morphological response in MDCK cells (Wells et al., 2002). Indeed, most of the reported morphological effects of PAK1 have been identified through expression of constitutively activated protein (Daniels et al., 1999; Manser et al., 1997), probably because wild-type proteins are rapidly turned off after stimulation. In contrast, over-expression of constitutively active PAK1 caused the formation of podosomes (Webb et al., 2005), a cellular structure related to invadopodia. Moreover, constitutively active PAK2 has been noted to produce podosomes that later dispersed throughout the cytoplasm of endothelial cells (Zeng et al., 2000). Thus, whilst over-expression

of normal WT-PAK1/2 is insufficient to enhance invadopodia formation, constitutively active PAK1 might be able to induce MCF-7 cells to form invadopodia. However, neither HGF nor DMOG could induce invadopodia formation in these cells **(chapter 3 and 4)**. Indeed, others have recently reported that invasive ability of MCF-7 cells could be increased by TGF-β signalling (Rosman et al., 2008; Walsh and Damjanovski, 2011). This suggests that the invasiveness of these could still be detected but not necessarily via the induction of invadopodia formation.

Both HGF and DMOG can enhance invadopodia formation in MDA-MB-231 cells. However, whilst PAK1 and PAK2 are required for the HGF induced response, DMOG was still able to induce invadopodia activity in knockdown cells. These suggest that HGF-mediated invadopodia activity is specifically regulated through PAK1 and PAK2 signalling modes and others have already reported a specific role for HGF in invadopodia formation (Rajadurai et al., 2012). In contrast, the increased levels of HIF-1α in DMOG treated cells were able to initiate invadopodia formation in the absence of PAK1/PAK2 expression, thus suggesting the involvement of a broader cytoskeletal signalling cascade or parallel signalling pathways. This is possibly mediated through the ability of HIF-1α to rapidly up-regulate expression of large number of different genes.

This chapter has identified PAK1 and PAK2 as two proteins required for invadopodia formation in MDA-MB-231 cells. Interestingly, it is also shown here that PAK1 and PAK2 have non-overlapping functions during invadopodia formation. PAK1 and PAK2 are both localised at invadopodia F-actin structures and sites of gelatin degradation. Moreover, loss of PAK1 or PAK2 expression inhibits HGF-induced invadopodia formation. In addition, PAK1 or PAK2 expression is not essential during DMOG-induced invadopodia formation.

Chapter 6 – β-PIX expression is up-regulated in response to over-expression of HIF-1α

Chapter 6 – β-PIX expression is up-regulated in response to over-expression of HIF-1α

6.1 Introduction

Previous chapters have demonstrated that invadopodia formation in MDA-MB-231 cells can be modulated by growth factor stimulation, exposure to hypoxia and changes in the level of PAK1 and PAK2 expression. Whilst PAK1 and PAK2 were identified as key components of HGF-mediated invadopodia induction, the mechanism of HIF-1α-induced induction is less clear. This chapter focussed on exploring further how increased levels of HIF-1α might directly contribute to invadopodia formation. HIF-1α is a transcription factor and thus it is likely that HIF-1α induced invadopodia formation might at least in part be mediated through changes in gene expression. Many proteins have been implicated in regulating the formation of invadopodia, including members of the Rho family GTPases Rac and Cdc42 (Nakahara et al., 2003), p-21-activated kinases (Ayala et al., 2008) and proteins also associated with other forms of cell: substratum adhesion such as paxillin and N-WASP (Badowski et al., 2008; Yamaguchi et al., 2005). Expression analysis has revealed that a number of genes which regulate cancer invasion such as CXCR4, pyruvate dehydrogenase kinase 1 (PDK1), matrix metalloproteinase 2, urokinase plasminogen activator receptor (uPAR), and fibronectin 1 are regulated by HIF-1α (Krishnamachary et al., 2003; Victor et al., 2006). Indeed, it has recently been shown that HIF-1α can modulate cell migration via induction of JMY (Coutts et al., 2011) and RhoE expression (Zhou et al., 2011). However, the regulation of cytoskeletal genes by HIF-1α has not been fully elucidated.

6.2 Results

6.2.1 PAK1/2 expression and activity are not regulated by HIF-1α

PAK1 and PAK2 are required for invadopodia formation and loss of expression attenuates the effect of HGF and DMOG treatment **(figure 5.7).** Therefore, the level of endogenous PAK1/2 protein expression was assessed in both DMOG treated and HIF-1α over-expressing cells. There was no detectable change in PAK1/2 expression level observed with either DMOG or HIF-1α over-expression **(figure 6.1a and b).** In parallel, the effects on PAK1/2 activity downstream of both DMOG and HIF-1α over-expression was also monitored, however no reproducible changes in PAK1/2 activity was observed (data not shown). This suggests that neither PAK1/2 expression levels nor activity are directly regulated during HIF-1α-driven invadopodia formation and cannot account for the changes in invadopodia activity detected.

6.2.2 Expression of cytoskeletal genes are regulated by HIF-1α

Given that no changes in PAK1/2 expression or activity were detected, an alternative approach was adopted. One of the most reliable and reproducible tools for analysing the changes in expression levels of a focused panel of genes is by PCR array. The particular array that was employed in this study was the pathway-focused, 96-well plate PCR Array which includes polymerases and primers tailored for cytoskeletal-related genes (Qiagen). MDA-MB-231 cells treated with DMOG and control (non-treated) were used for this study. Data analysis from the real-time-qPCR was performed by normalizing the raw data to the housekeeping genes provided by the manufacturer (SABiosciences). The internal positive controls of the PCR-array are the genes well characterised to be regulated by HIF-1α such as VEGFA, c-Met and EGFR. Unsurprisingly, these genes were significantly up-regulated by DMOG. The top 10 up-regulated genes are listed in **Table 6.1**. Of these genes, Arf6, uPAR, Cdc42, and Arp2 are already known to be involved in the formation of invadopodia (Furmaniak-Kazmierczak et al., 2007; Tague et al., 2004; Yamaguchi et al., 2005). Although, this is the first report of a direct link between HIF-1α stabilisation and protein expression levels for these hits. In addition, expression of β-PIX, a cytoskeletal protein not yet known to be involved in the formation of invadopodia,

was also elevated in the PCR array. β-PIX is associated with regulation of adhesion turnover (Kuo et al., 2011; Rosenberger and Kutsche, 2006; Stofega et al., 2004) and was therefore selected for further studies.

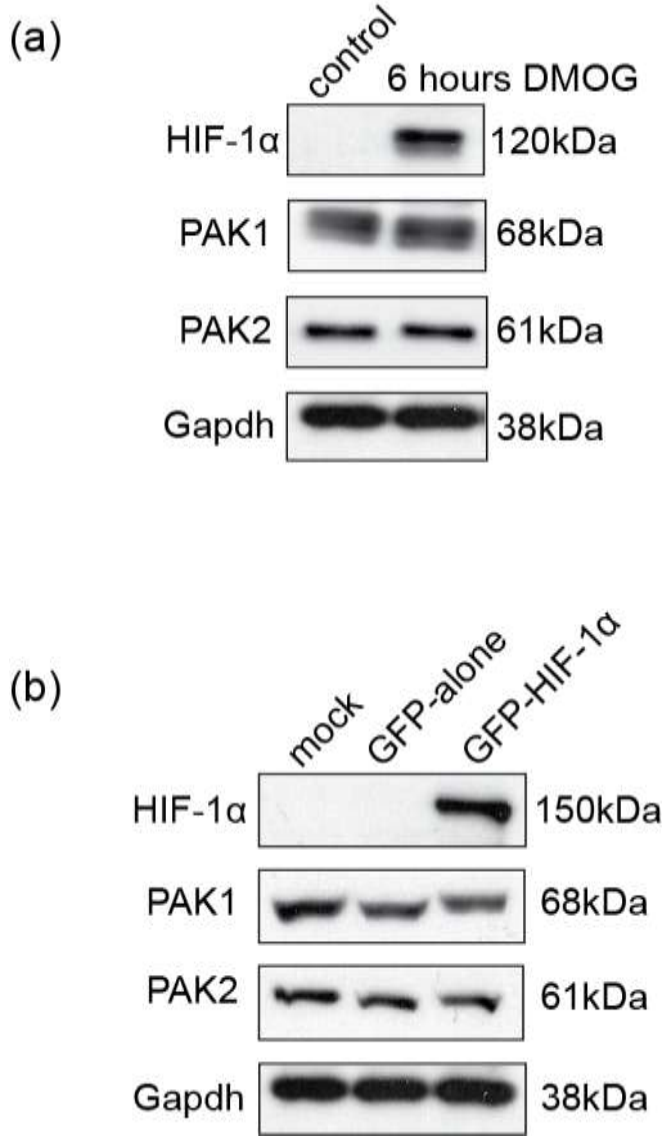

Figure 6.1 PAK1 and PAK2 expression levels do not change in response to DMOG or HIF-1α over-expression. a) MDA-MB-231 cells were seeded at $3X10^4$ cells/ml and allowed to adhere for 24 hours. Cells were treated with dimethyloxaloylglycine (DMOG) for 6, 8 and 6 hours plus maintaining DMOG for an extra 2 hours. **b)** Cells were seeded at $1X10^5$ cells/ml in a 6-well plate and transfected with GFP-HIF-1α. Cells were lysed at 24 hours following transfections. Equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of PAK1 and PAK2 (Cell Signaling Technology) antibodies. ERK and Gapdh were used as loading controls. These results are representative of three independent experiments.

Up-regulated gene	Symbol	p value	Fold Change	Significance
ADP-ribosylation factor 6	ARF6	0.00046	1.5	**
Plasminogen activator, urokinase receptor	UPAR	0.00074	2.9	**
Rho guanine nucleotide exchange factor 7	β-PIX	0.0015	2.0	**
Vascular endothelial growth factor A	VEGFA	0.0014	6.0	**
WAS protein family, member 2	WAVE2	0.0013	1.8	**
Cell division cycle 42 (GTP binding protein)	CDC42	0.016	1.6	*
Epidermal growth factor receptor	EGFR	0.017	1.7	*
Hepatocyte growth factor receptor	C-MET	0.034	1.6	*
Actin-related protein homolog 2	ARP2	0.007	1.4	*
Integrin, beta 3	ITGβ3	0.02	2.1	*

Table 6.1 Top 10 hits of up-regulated cytoskeletal genes in response to 6 hours DMOG treatment. MDA-MB-231 cells were seeded at $1X10^5$ cells/ml. and allowed to adhere for 24 hours. Cells were treated with dimethyloxaloylglycine (DMOG) at a concentration of 0.5mM for 6 hours. RNA was extracted from the treated and non-treated (control) cells. RNA was converted into cDNA (see materials and methods) and used in RT^2 Profiler™ PCR Array specific for Human Cell Motility gene profiling. The top 10 hits have been listed in this table. Full results can be referred to in the appendix. These results are representative of two independent experiments. Statistical significance compared with control (non-treated) was calculated using Student's *t*-test; *, $P<0.05$. **, $P<0.005$.

6.2.3 β-PIX and VEGF mRNA are up-regulated in response to increased HIF-1α expression

Initially, to confirm the data from PCR-Array studies, reverse transcriptase PCR (RT-PCR) was used to monitor changes in β-PIX and VEGF mRNA expression levels. Level of β-PIX and VEGF mRNA expression significantly increased in response to DMOG treatment **(figure 6.2 a and c).** These results confirmed the results observed in the PCR-array. In parallel with DMOG treatment, RT-PCR was also performed on cells over-expressing GFP-HIF-1α. The level of β-PIX and VEGF mRNA expression was also significantly up-regulated by the HIF-1α over-expression system **(figure 6.2 b and d).** This indicates that the over-expression strategy is functional and more importantly specific over-expression of HIF-1α significantly up-regulated transcription of the same genes as had been observed in response to DMOG treatment.

6.2.4 β-PIX protein expression level increases in response to DMOG and HIF-1α over-expression

The effect of DMOG treatment in enhancing invadopodia forming potential of MDA-MB-231 cells was observed at 6 hours post-treatment **(figure 4.2).** The level of β-PIX mRNA expression significantly increased at 6 hours DMOG treatment **(Figure 6.2 a and c)** and β-PIX protein levels were also significantly increased, in cells treated with DMOG for 6 hours **(figure 6.3 a).** Alongside DMOG-treatment, β-PIX protein expression was also monitored in cells over-expressing GFP-HIF-1α and in cells incubated for 6 hours in a hypoxia incubator. In both conditions, β-PIX protein expression was significantly increased **(figure 6.3 b and c).**

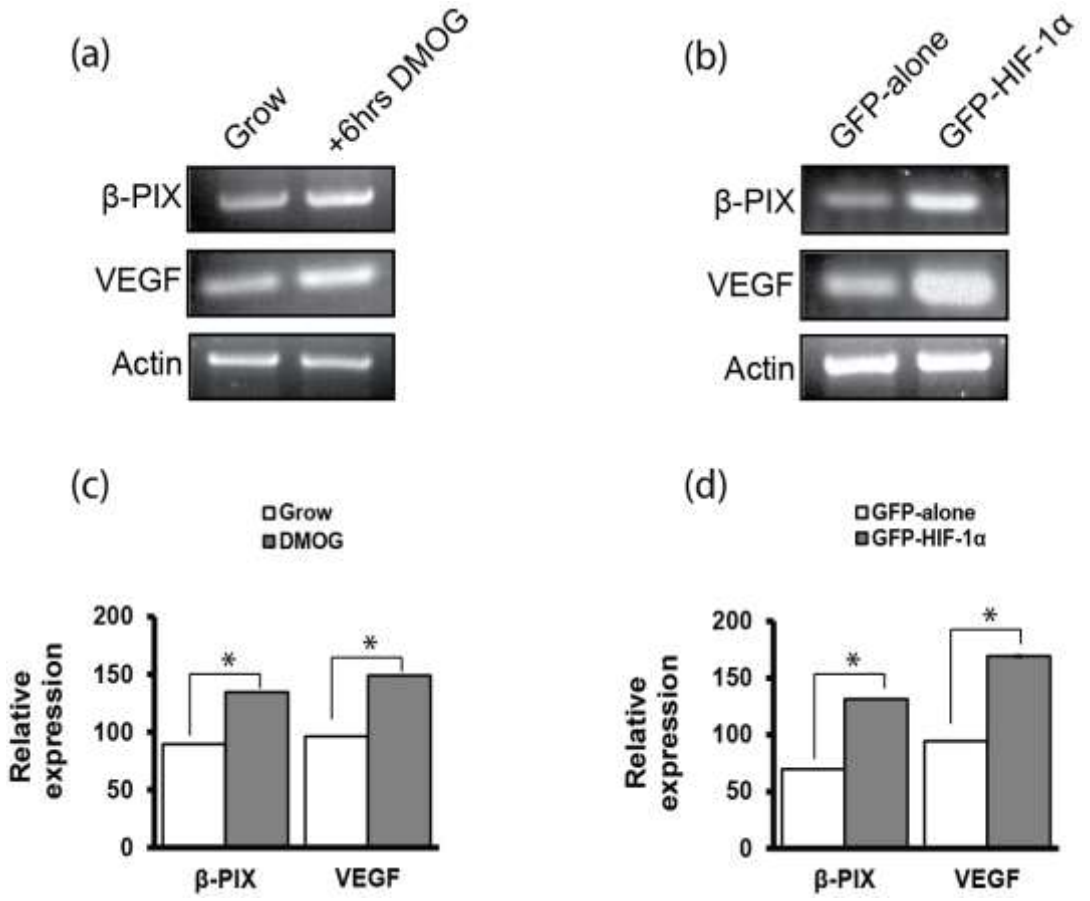

Figure 6.2 β-PIX and VEGF genes are up-regulated in response to DMOG or over-expression of HIF-1α. (a) MDA-MB-231 cells were seeded at $3X10^4$ cells/ml and allowed to adhere for 24 hours. Cells were either treated with dimethyloxaloylglycine (DMOG) for 6 hours or non-treated (control). **(b)** Cells were seeded at $1X10^5$ cells/ml in a 6-well plate and transfected with GFP-alone (control) and GFP-HIF-1α using Lipofectamine 2000 transfection reagent (see materials and methods). Total RNA was extracted from the cells in all conditions. RNA was then converted into cDNA and used in PCR using primers specially designed for β-PIX, VEGF and actin as PCR loading control (see materials and methods). Following PCR, reaction samples were equally loaded on an agarose gel and run for the required amount of time. The bands were viewed using a UV Transilluminator machine. **(c and d)** Densitometric analysis of relative β-PIX, VEGF and actin expression. The results shown are RT-PCR from each experimental condition over three separate experiments. Statistical significance compared with control (non-treated) or/and GFP-alone was calculated using Student's *t*-test; *, $P<0.05$.

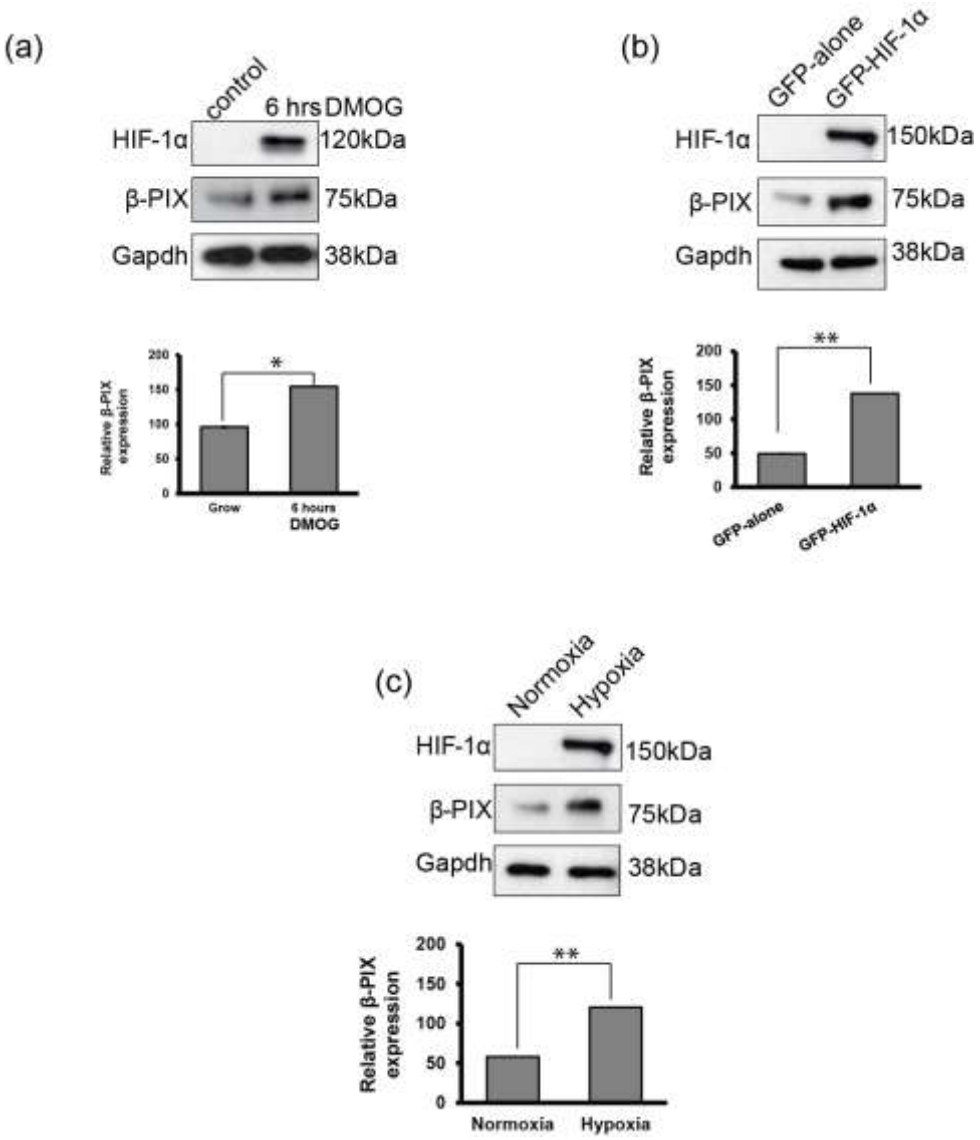

Figure 6.3 MDA-MB-231 cells increase β-PIX protein expression in response to hypoxic conditions. (a) Cells were seeded at $3X10^4$ cells/ml and allowed to adhere for 24 hours. Cells were stimulated with dimethyloxaloylglycine (DMOG) for 6, 8 and 6 hours plus maintaining DMOG for an extra 2 hours. **(b)** Cells were seeded at $1X10^5$ cells/ml in a 6-well plate and transfected with GFP-HIF-1α using Lipofectamine 2000 transfection reagent. **(c)** Cells were seeded at $2X10^4$ cells/ml on plastic dishes and allowed to adhere for 24 hours. Cells were exposed to normoxia (20% oxygen) and hypoxia (1% oxygen) for 16 hours. Cells were lysed at 6 or 8 hours DMOG treatment, 24 hours following transfections, and 16 hours hypoxia/normoxia, and equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of HIF-1α (Cell Signaling Technology), and β-PIX (Cell Signaling Technology) antibodies. Gapdh, a house-keeping protein was used as a loading control. These results are representative of three independent experiments. **(a, b and c bar graphs)** Relative expression of β-PIX was measured using densitometric analysis. The results shown are mean ±SEM from each experimental condition over three separate experiments. Statistical significance compared with control (non-treated) or GFP-control or normoxia was calculated using Student's *t*-test; *, $P<0.05$. **, $P<0.005$.

6.2.5 The β-PIX gene contains two putative HREs

One pre-requisite of gene transcription is the binding of the HIF-1 complex to hypoxia response elements (HREs) in the promoter region of its target genes. In order to confirm whether β-PIX could potentially be one of the target genes of HIF-1, HREs were located in the promoter sequence of β-PIX. Computer-assisted analysis for the inspection of the upstream promoter region of β-PIX identified two regions containing the core sequence of a consensus (HREs) 5'-RCGTG-3'(R, purine [A or G]) located between -651 and +299 sequence in the β-PIX promoter. In these regions, two putative HREs underlined in red (CGTG) were found **(figure 6.4).**

6.2.6 β-PIX is required for invadopodia formation

β-PIX has been reported to be an important protein involved in cell motility and invasion, and more importantly shown for the first time here, is up-regulated by increased HIF-1α expression, thus the effects of β-PIX on invadopodia formation were investigated. β-PIX expression was successfully depleted by 80% after 24 hours RNAi transfection using siRNA SMARTpool technology targeting four mRNA regions at once **(figure 6.5 a and b).** β-PIX-depleted MDA-MB-231 cells were then seeded on gelatin-coated coverslips to assay for matrix degradation. The percentage of cells forming invadopodia dramatically dropped to 10% when β-PIX expression was reduced **(figure 6.5 c).** Moreover, β-PIX depletion attenuated the DMOG response in which percentage of cells forming invadopodia was significantly reduced compared to control cells with DMOG alone **(figure 6.5 b and c).** Furthermore, when β-PIX knockdown cells were transfected with GFP-HIF-1α, the level of invadopodia formation did not reach siRNA control levels **(figure 6.6)**. However, both DMOG and HIF-1α over-expression still induced some invadopodia formation in the β-PIX knockdown background **(figure 6.5 and 6.6).** This suggests that HIF-1α drives invadopodia not just via a β-PIX pathway, but also via other signalling mechanisms.

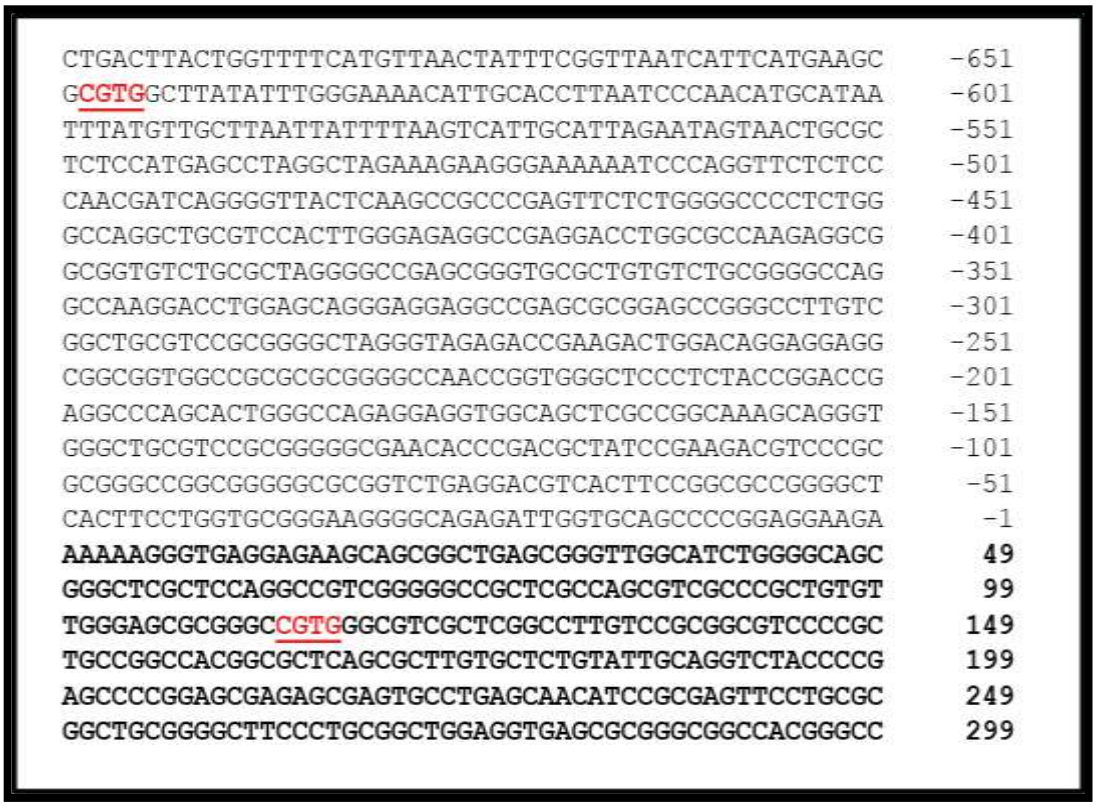

Figure 6.4 Two putative hypoxia-response elements are located in the promoter sequence of β-PIX. The HREs for β-PIX were identified using a gene promoter search website **http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=searchPromForm.** Following the identification of the sequence of the promoter, word search for CGTG was performed. The putative HREs are shaded in red and underlined. Bold shading indicates exon 1.

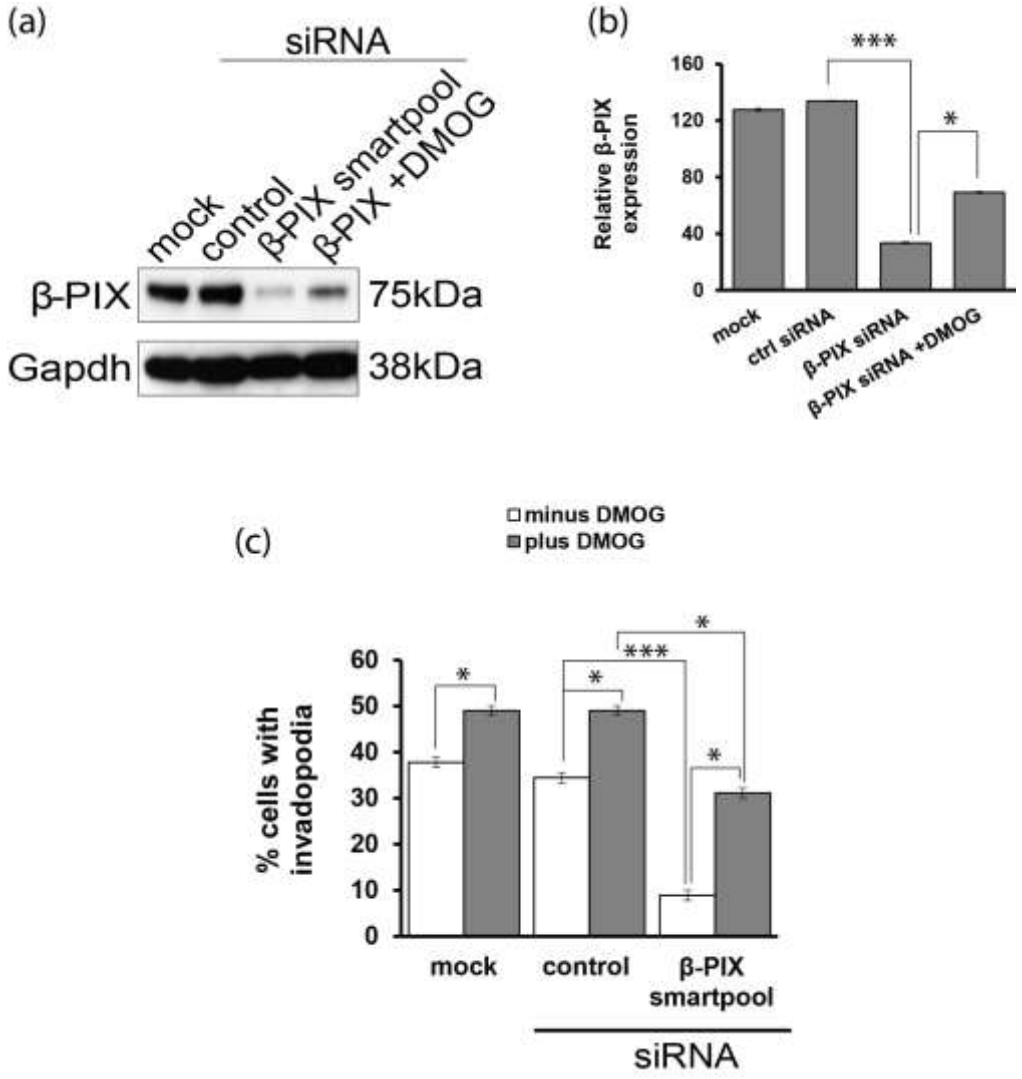

Figure 6.5 Loss of β-PIX expression influences invadopodia formation and attenuates the DMOG response in MDA-MB-231 cells. (a) Cells were seeded at $3X10^4$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with mock, control and β-PIX siRNAs for 24 hours, with and without stimulation with DMOG for 6 hours. Cells were lysed for 10 minutes in lysis buffer. Equal amounts of protein lysates were electrophoresed on 7.5% SDS polyacrylamide gels. Western blot of the lysates was probed for levels of β-PIX (Cell Signaling Technology). Gapdh, a house-keeping protein was used as a loading control. **(b)** Relative expression of β-PIX was measured using densitometric analysis. **(c)** β-PIX siRNA-treated cells, with and without DMOG were seeded on gelatin-coated coverslips. Cells were scored for the presence of actin puncta that colocalize with area of degradation on the gelatin and the mean percentage of cells with invadopodia formation calculated. **(d)** The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with control siRNA or β-PIX siRNA was calculated using Student's *t*-test; *, P<0.05. ***, P<0.0005.

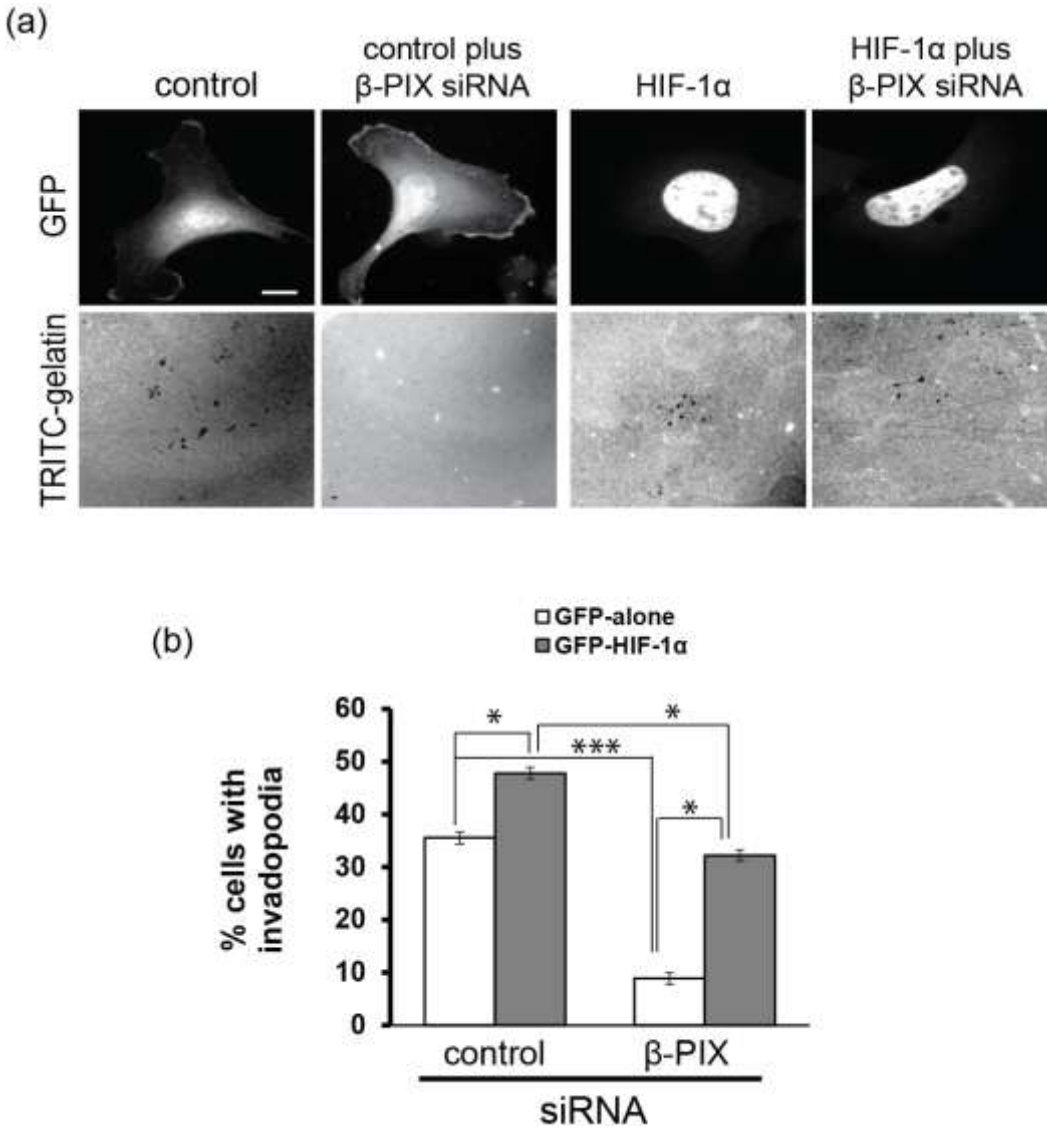

Figure 6.6 Loss of β-PIX expression influences HIF-1α-dependent invadopodia formation in MDA-MB-231 cells. (a) Cells were seeded at $1X10^5$ cells/ml and allowed to adhere for 24 hours. Cells were transfected with GFP-control and GFP-HIF-1α plasmids for 24 hours, followed by transfection with β-PIX smartpool siRNA oligo for another 24 hours. These cells were then re-seeded on gelatin-coated coverslips. Cells were scored for the presence of dark holes on the gelatin only within the cell margin and the mean percentage of cells with invadopodia formation calculated. Scale bar=10μm. **(b)** The results shown are mean ±SEM of 30 cells from each experimental condition over three separate experiments. Statistical significance compared with either GFP-control or GFP-HIF-1α was calculated using Student's *t*-test; *, P<0.05. ***, P<0.0005.

6.3 Discussion

Chapter 5 demonstrated that PAK1/2 are not only required for invadopodia formation but are also required during DMOG-induced invadopodia formation. These results suggested that PAK1/2 could potentially be a novel transcriptional target of HIF-1α. Thus the level of expression and activity of PAK1/2 in response to DMOG and HIF-1α over-expression was monitored. However, HIF-1α did not regulate the expression or activity of PAK1/2 **(figure 6.1).** Given that HIF-1α does not appear to directly modulate PAK1/2 invadopodia it may up-regulate other cytoskeletal proteins to promote invadopodia formation and matrix degradation. As a transcription factor HIF-1α is known to be associated with a large number of targets, many of which are associated with angiogenesis.

This study found that VEGFA and c-Met expression levels were significantly increased (Table 6.1) in breast cancer cells when HIF-1α protein levels were increased. Other cytoskeletal genes such as Cdc42, Wave2, Arp2 and β-PIX were also identified as being HIF-1α responsive (Table 6.1). This is the first time that these proteins have been identified as potentially HIF-1α regulated. It will be interesting, in the future, to explore in more detail the relationship between HIF-1α stabilization during hypoxia and the regulation of the expression of proteins associated with migration and invasion. Among the invasion-related genes known to be transcriptionally up-regulated by HIF-1 is urokinase-type plasminogen activator receptor (uPAR) which promotes invasion of breast (Yoon et al., 2006), pancreatic and liver cancers (Buchler et al., 2009). Previous reports suggested that over-expression of HIF-1α has been observed in the invasive front of human brain and colon cancer biopsies (Zagzag et al., 2000; Zhong et al., 1999), where uPAR expression is localised. Putative HIF-1 binding sites are located upstream of the uPAR promoter (Graham et al., 1998), and uPAR expression and in vitro invasiveness of human colon carcinoma cells are increased by HIF-1α over-expression, and decreased by knockdown of HIF-1α (Krishnamachary et al., 2003). Similarly, human renal cell carcinoma cells lacking VHL (increase HIF-1α level) show constitutively elevated uPAR levels, while mouse embryonic stem cells deficient for HIF-1α expression fail to upregulate uPAR expression under hypoxic conditions (Krishnamachary et al., 2003).

Other studies have demonstrated that HIF-1 also regulates adhesion and migration of melanoma cells through the regulation of alphavbeta3 (αvβ3) integrin surface expression at the plasma membrane (Cowden Dahl et al., 2005). In addition, a study highlighted the ability of HIF-1 to enhance the transcription of the Met receptor and promote hepatocyte growth factor-dependent invasion in hypoxic areas of tumours (Pennacchietti et al., 2003). These effects are mediated by HIF-1 binding sites located in the 5' untranslated region of the *Met* promoter (Pennacchietti et al., 2003). Another study suggested the requirement of HIF-1α expression for the synergistic activities of hypoxia and HGF on cellular invasion (Hara et al., 2006).

Increased β-PIX protein expression, following stabilisation of HIF-1α was validated and the increased levels correlated in time with increased levels of invadopodia formation in the cell population. If β-PIX is a HIF-1 target gene, hypoxia-responsive-elements within the β-PIX proximal promoter are required and two putative HREs in the β-PIX promoter sequence were identified. Other ways to further establish the functional significance of HIF-1α in regulating β-PIX transcription is by employing a gel mobility shift assay (EMSA), a luciferase reporter assay to measure luciferase activity and chromatin immunoprecipitation (ChIP) to explore the recruitment of HIF-1α to the β-PIX promoter.

One of the key cytoskeletal proteins, the Arp2/3 complex is important for a variety of specialized cell functions that involve the actin cytoskeleton and is already known for its requirement during invadopodia formation in breast cancer cells (Yamaguchi et al., 2005). Indeed many proteins have been implicated in regulating the formation of invadopodia, including the members of the Rho family GTPases Rac and Cdc42 (Nakahara et al., 2003), p21-activated kinases (PAKs) (Ayala et al., 2008)and proteins also associated with other forms of cell:substratum adhesion such as paxillin and N-WASP (Badowski et al., 2008; Yamaguchi et al., 2005). However, β-PIX, a guanine nucleotide exchange factor (GEF) for Rac and Cdc42, has been implicated in the regulation of cell adhesion (Kuo et al., 2011; ten Klooster et al., 2006) but has not been implicated in invadopodia formation.

The N-terminus of PAK1 contains multiple proline-rich sites that bind to src homology 3 (SH3) domain-containing proteins, including β-PIX (Manser et al., 1998). β-PIX specifically binds to PAK1/2 (Bagrodia et al., 1998; Manser et al., 1998). PAK1 has been associated with focal complexes which represent sites of attachment with the substratum (Manser et al., 1997). β-PIX has been implicated in the localization of PAK1 to focal adhesions, because disruption of PIX-binding proline-rich regions abrogated the ability of ectopically expressed PAK1 to localize to focal adhesions (Manser et al., 1998). β-PIX was found in a complex with PAK1 (Stofega et al., 2004; Webb et al., 2005), a protein thought to regulate invadopodia formation (Ayala et al., 2008) and is shown in Chapter 5 to be required for invadopodia formation in MDA-MB-231 cells. Thus invadopodia formation induced by HIF-1α might be mediated at least in part through the activity of β-PIX.

In support of this hypothesis, reduced β-PIX expression levels led to a dramatic reduction in invadopodia formation. This would suggest a fundamental requirement for β-PIX expression during invadopodia formation and matrix degradation. Furthermore, in a background of β-PIX knockdown, neither DMOG treatment nor GFP-HIF-1α over-expression were able to induce invadopodia formation to the same extent as control cells**.** However, the modest induction in invadopodia formation in the β-PIX knockdown background might be due to the partial restoration of β-PIX expression following DMOG addition **(see lane 4 Figure 6.5a)**. Again, this demonstrates that β-PIX expression levels are highly sensitive to levels of HIF-1α in cells. Alternatively, DMOG/ HIF-1α over-expression induced increase in invadopodia activity in a β-PIX knockdown background might indicate that HIF-1α is acting through multiple cytoskeletal pathways to induce invadopodia formation, a hypothesis that is supported by the PCR Array studies. Nonetheless, this study points to an essential requirement for β-PIX.

A role for β-PIX in invadopodia formation has not been previously reported but PIX family members have been implicated in the regulation of podosome formation (Gringel et al., 2006; Webb et al., 2005). Podosomes are highly dynamic, actin-based structures that function at sites of cell adhesion and active ECM remodelling (Linder and Aepfelbacher, 2003). β-PIX has been reported to promote podosome formation where it colocalized with actin columns and enhanced the formation of these

structures in vascular smooth muscle cells (Webb et al., 2005). Interaction between PAK1 and β-PIX was required for podosome formation. Podosome biogenesis requires two highly coordinated events involving the remodelling of both focal complexes and actin stress fibers. It has been suggested that PAK-PIX interaction plays a crucial role in this regulatory mechanism (Webb et al., 2005).

β-PIX has been implicated in influencing the migration and invasive activity of Src transformed cells (Feng et al., 2010). A number of reports suggested that β-PIX localizes to focal complexes (Nayal et al., 2006; Zhao et al., 2000). It would be interesting to determine whether β-PIX localises to invadopodia or can be recruited to invadopodia in response to hypoxia. Furthermore, the development of β-PIX antibodies suitable for immunohistochemistry would facilitate a better understanding of how β-PIX levels are regulated in hypoxic tumours.

Taken together, this study demonstrates an important and novel link between β-PIX and HIF-1α, where β-PIX is identified as a novel HIF-1α target gene that mediates invadopodia formation in breast carcinoma cells. The ability of HIF-1α to elevate β-PIX expression provides a mechanism that could maintain β-PIX at sufficient concentrations in hypoxic cells to facilitate cell invasion. Moreover, this study identified a cohort of cytoskeletal genes whose expression is regulated by HIF-1α. Given the enhanced cell motility and invasion that occurs under hypoxia (Sullivan and Graham, 2007), it makes sense that cytoskeletal proteins involved in focal adhesion turnover, cell motility and invadopodia formation, like β-PIX, should be responsive to such stimuli.

Chapter 7 – Concluding remarks

Chapter 7 – Concluding remarks

This study has demonstrated that extracellular cues by HGF stimulation **(Chapter 3)** and hypoxia (resulting in an increase in the level of hypoxia-inducible transcription factor HIF-1α) affect invadopodia forming potential of MDA-MB-231 breast carcinoma cells **(chapter 4)**. Moreover, this is the first report to suggest a link between HIF-1α and invadopodia formation in MDA-MB-231 breast carcinoma cells. At the molecular level PAK1 and PAK2 were specifically localised at invadopodia and loss of PAK1 or PAK2 expression inhibited HGF-induced invadopodia formation **(Chapter 5)**. Moreover, it was established that PAK1 and PAK2 have non-overlapping functions during invadopodia formation. Furthermore, β-PIX was identified as a novel HIF-1α target gene that mediates invadopodia formation **(Chapter 6)**.

Role of PAK1 and PAK2 in invadopodia formation

This study and others (Rajadurai et al., 2012; Tague et al., 2004) have reported that HGF can induce the formation of invadopodia in cancer cells and HGF is known to stimulate the activity of PAK kinases (Royal et al., 2000). Indeed, HGF stimulation increases PAK1 and PAK2 phosphorylation at Thr423/Thr402 on cells adhered on gelatin substrate **(Chapter 5)** and PAK1 and PAK2 are required for HGF-induced invadopodia formation. HGF stimulates PAK1/2 autophosphorylation **(Chapter 5),** PAK phosphorylation of cortactin is thought to promote invadopodia activity (Webb et al., 2006) and the PAK1-AID inhibits invadopodia formation (Ayala et al., 2008). Over-expression of kinase-active or kinase-dead PAK1 drives podosome formation suggesting that PAK1 exhibits kinase-dependent and kinase- independent functions. Therefore in the future, it might be interesting to more closely examine the role of PAK kinase activity during invadopodia formation in MDA-MB-231 cells. Expression of kinase dead rescue constructs would better elucidate the role of kinase activity, whilst other domain mutants could explore the role of proline rich binding interactions and/or Rho family GTPase activation.

Since PAK1 and PAK2 appear to play important roles in invadopodia formation, it is intriguing to speculate which downstream effectors or signalling cascades the PAKs

may be influencing. From the current literature it is known that PAK1 can phosphorylate LIMK1 (Edwards et al., 1999) and that activated LIMK1 in turn induces phosphorylation of cofilin, an actin filament-depolymerization protein that is inactivated by LIMK1 phosphorylation (Arber et al., 1998). Furthermore, LIM kinase has been implicated in invadopodia formation (Scott et al., 2010). Similarly, cofilin has been linked to invadopodia formation and has been shown to localize at invadopodia (Yamaguchi et al., 2005). Expression of constitutively active cofilin up-regulates the expression of the matrix-degrading enzymes matrix metalloproteinase 2 (MMP2) and 14 in melanoma cells (Dang et al., 2006). Moreover, expression of both the serine protease urokinase type plasminogen (uPA) and its receptor uPAR are higher in the breast cancer cell line MDA-MB-435 upon LIMK1 over-expression (Bagheri-Yarmand et al., 2006). uPA is also a matrix-degrading protease and an activator of multiple other matrix proteases, including MMPs and transcriptional up-regulation of uPAR in MDA-MB-231 cells was found under hypoxic conditions **(Chapter 6)**. It is therefore likely that PAK1 and maybe PAK2 could affect invadopodia by phosphorylating LIMK which in turn phosphorylates cofilin, to regulate recruitment of MMPs facilitating ECM degradation.

PAK1/2 might regulate F-actin levels by influencing cofilin activity as outlined above or by phosphorylation of cortactin (Webb et al., 2006). The enrichment of Tyr-phosphorylated cortactin in invadopodia is a hallmark of functional invadopodia (Bowden et al., 2006). In resting platelets, PAK1/2 and cortactin form a complex. Upon thrombin stimulation, this complex dissociates and releases cortactin to the periphery of the cell with a concomitant activation of PAK (Vidal et al., 2002). PAK1 has been implicated in cortactin phosphorylation and this subsequent phosphorylation is important during invadopodia maturation whereby recruitment of MT1-MMP takes place to facilitate ECM degradation. PAK1 has also been shown to induce podosome formation in vascular smooth muscle cells and it is partially co-localised with cortactin in podosomes. PAK1 phosphorylation of cortactin may play a role in the regulation of the dynamics of branched actin structures during invadopodia formation; consistent with a finding that PAK-kinase activity increased the turnover of podosomes.

The non-redundancy in the roles of PAK1 and PAK2 in invadopodia formation is very interesting, although other studies have shown examples of non-overlapping roles between these closely related proteins (Bright et al., 2009; Coniglio et al., 2008). The functions of PAKs have been elucidated through the identification of protein binding partners and kinase substrates. To reveal the full scope of PAK1 and PAK2 in invadopodia formation, it will be important to identify the downstream effectors of PAK1/2 that are responsible for specific phenotypic alterations that lead to invasive state of cancer cells. Although PAK1 and PAK2 are thought to share essentially indistinguishable substrate specificity, interaction with cortactin (Vidal et al., 2002) and p41-Arc (Vadlamudi et al., 2004) is unique to PAK1 and has not been related to PAK2. Recently, PAK1 phosphorylation was shown to increase the interaction of cortactin with N-WASP to mediate endocytosis (Grassart et al., 2010). It could be speculated that PAK1 may regulate invadopodia formation via a similar mechanism.

Almost all PAK2 binding partners have also been shown to bind PAK1; however, the exact effectors and mechanism of activation of PAK2-induced cytoskeletal activities have not been clearly elucidated. Furthermore, little is known about the regulatory mechanism of the PAK2 pathway. Studies have established that myosin II RLC is a substrate for PAK2 (Chew et al., 1998; Ramos et al., 1997) and PAK2 phosphorylation of myosin II is thought to play a role in endothelial cell retraction (Zeng et al., 2000). In addition, a recent study has identified MYO18A, a member of the myosin superfamily as a novel interacting partner of the PAK2/β-PIX/GIT1 complex and suggested its potentially important role in modulating focal adhesion turnover (Hsu et al., 2010). Furthermore, it has also been proposed that force and membrane tension generated by intracellular motors (such as myosin) and regulated by substrate viscoelasticity might affect actin polymerization, and in turn the formation of invadopodia (Albiges-Rizo et al., 2009). It is reasonable to speculate that involvement of PAK2 in invadopodia formation may be via this mechanism. Based on this speculation, it can be hypothesised that PAK1 may act as a scaffolding protein that couples to downstream effectors mediating assembly of molecules to promote invadopodia formation. Furthermore, PAK1 could be phosphorylating downstream substrates involved in invadopodia formation whereas PAK2 might be

involved in the local modulation of contractile forces that are all likely to play a central role in the promotion of invadopodia.

β-PIX interplay with PAK1/2 during invadopodia formation

In addition to identifying PAK1 and PAK2 as key components of invadopodia formation in MDA-MB-231 cells, the depletion of β-PIX by siRNA significantly impaired invadopodia formation, and significantly reduced the induction of invadopodia downstream of HIF-1α stabilisation **(Chapter 6)**. These data revealed for the first time the importance of β-PIX activity during invadopodia formation. β-PIX is the guanine nucleotide exchange factor (GEF) for Cdc42 and Rac that can form larger complexes with various proteins including PAK1, PAK2 (Manser et al., 1998; Shin et al., 2002; ten Klooster et al., 2006), Rac/Cdc42 (Shin et al., 2004), GIT (Zhao et al., 2000) and Shank (Park et al., 2003). Indeed PAK2 phosphorylates β-PIX in response to bFGF during bFGF-induced neurite extension (Shin et al., 2002).

β-PIX activity has already been associated with HGF stimulation where a β-PIX/GIT1/ WAVE2 complex was localised at the leading edge of the cells, which promoted lamellipodia formation (Morimura et al., 2009). In a separate study HGF induced lamellipodia formation required Rac1 and PAK1 for the transport of WAVE2 along the microtubules to the leading edge (Takahashi and Suzuki, 2008). Here, PAK1 plays a critical role in the induction of WAVE2 transport along microtubules and lamellipodia formation by mediating phosphorylation of tubulin-bound stathmin/Op18 at Ser38 and subsequent recruitment of the protein to a PAK1–WAVE2–kinesin complex (Takahashi and Suzuki, 2009). Collectively, these findings suggest the possible involvement of both β-PIX and PAK in the regulation of WAVE2 transport, which consequently leads to lamellipodia formation in MDA-MB-231 cells. WAVE2 was also transcriptionally upregulated by HIF-1α **(Chapter 6)**. Therefore, β-PIX may act via WAVE2 to promote invadopodia formation downstream of increased HIF-1α expression. However, an association between invadopodia and WAVE2 has not been reported, indeed in MTLn3 cells WAVE2 is not required for invadopodia formation (Yamaguchi et al., 2005).

β-PIX has not been associated with invadopodia formation but it has been reported to mediate focal adhesion dynamics and podosome (structurally related to invadopodia) activity via its interaction with PAK and GIT1. GIT1 is an ADP-ribosylation factor GTPase-activating protein (ARFGAP), which specifically regulates ARF6 (Vitale et al., 2000; Zhao et al., 2000). Interestingly, ARF6 has been implicated in the formation of invadopodia downstream of HGF (Tague et al., 2004) and from the PCR array analysis, ARF6 has been shown to be transcriptionally up-regulated by HIF-1α **(Chapter 6)**. Moreover, ARF6 has a critical role in regulating the shedding of microvesicles containing functional proteases to facilitate ECM degradation (Muralidharan-Chari et al., 2009). An important function of GIT1 is that it serves as a signalling anchor between β-PIX, PAK and the focal adhesion complex in migrating cells (Brown et al., 2002). The over-expression of a β-PIX mutant that either cannot bind PAK1 or lacks GEF function blocks PAK1 activation (Ku et al., 2001) suggesting that β-PIX is required for Rho family GTPase activation upstream of PAK1. Abolition of the PIX-binding ability of PAK prevents both the translocation of PIX and GIT to focal adhesions and the formation of podosomes (Webb et al., 2005). The essential role for β-PIX in podosome formation is highlighted by the fact that over-expression of β-PIX by itself can induce the formation of podosomes. Given this close relationship between β-PIX and PAK in adhesion structures, and that in MB-MDA-231 cells, invadopodia formation requires PAK1, PAK2 and β-PIX expression it is reasonable to speculate that the requirement for PAK1 and PAK2 during HGF-induced invadopodia formation could involve interactions with β-PIX **(Figure 7.1)**. It will be interesting in the future to look at the recruitment/subcellular localisation of PAK1/PAK2 in β-PIX depleted cells. Moreover, can over-expressing WT PAK1/PAK2 or kinase active/dead mutants rescue the β-PIX phenotype?

Another possible mechanism of β-PIX action during invadopodia formation could be through the molecule Scrib. Scrib is a scaffold protein conserved during evolution. The β-PIX/PAK protein complex interacts with Scrib and is required for the retention of the protein complex at the leading edge of migratory cells. PAK1 and PAK2 isoforms are part of this complex in proportion that is relative to their abundance in the cells (Nola et al., 2008). Additionally, the chemotactic activity of heregulin (HRG) was dependent on the presence of Scrib, β-PIX and PAK, and on

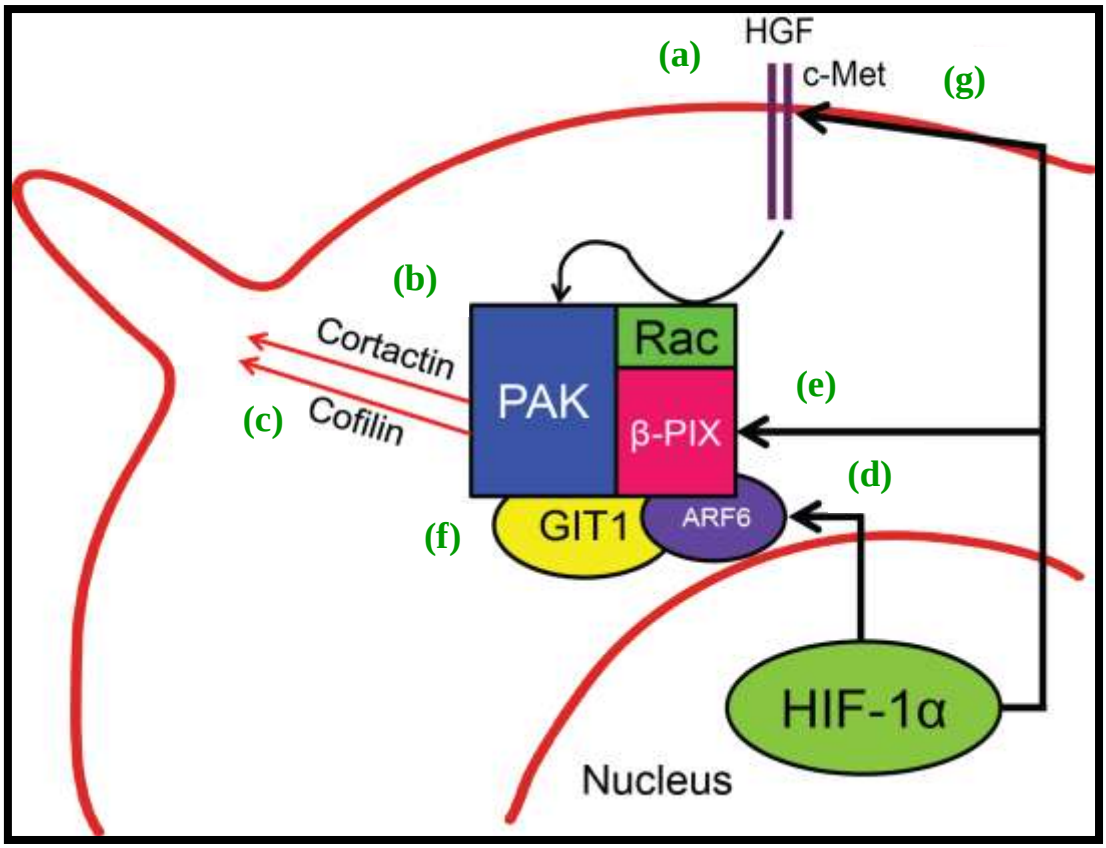

Figure 7.1 Proposed model for signalling pathways that promote invadopodia formation. **(a)** Upon HGF binding to c-Met, Rac is activated and this in turn could lead to PAK activation. **(b)** PAK can phosphorylate cortactin which could promote invadopodia formation via recruitment of MT1-MMPs. **(c)** Additionally, PAK could phosphorylate LIMK and that activated LIMK in turn induces phosphorylation of cofilin that has been linked to invadopodia formation. **(d)** HIF-1α is an important transcription factor that up-regulates known components of invadopodia such as Arf6. **(e)** Another transcriptional target of HIF-1α is β-PIX which is recognised as a GEF for Rac, an activator of PAK. β-PIX expression is required for invadopodia formation and loss of β-PIX expression attenuated HIF-1α-induced invadopodia formation in MDA-MB-231 breast carcinoma cells. **(f)** β-PIX may promote invadopodia formation by forming a complex with GIT1 and/or ARF6 since ARF6 has a critical role in regulating the shedding of microvesicles containing functional proteases. PAK1/PAK2 may be directly activated by interaction with β-PIX to facilitate invadopodia formation.The functional complex formed by β-PIX-PAK-GIT1-Arf6 could subsequently act to recruit and activate cofilin and/or cortactin to promote invadopodia formation. **(g)** HIF-1α is established as a transcriptional modulator of c-Met. Taken together, β-PIX could have the dual role of activating as a GEF and then functioning as an active scaffolding protein; bringing together components, each domain being necessary for the formation of invadopodia in MDA-MB-231 cells.

the interaction between Scrib and β-PIX-PAK. Cells lacking Scrib or β-PIX poorly activate PAK (low T423 phosphorylation) (Nola et al., 2008) suggesting that β-PIX is required for PAK activation. Thus Scrib may also be involved in invadopodia formation; however, further studies will be needed to address this hypothesis.

Future studies on invadopodia formation

Detailed analysis of invadopodia has resulted in a much clearer definition of these cellular organelles. They represent a powerful paradigm to study the tight interactions between the cytoskeleton, signalling and trafficking machineries. Beyond completion of the molecular and cellular description of invadopodia, a challenging issue will be to monitor matrix remodelling and invadopodia dynamics in invasive cells in the tumour microenvironment. High spatial- and temporal-resolution imaging techniques are available that allow visualization of invadopodia components in action in living cells in 3D reconstituted matrices and recent developments in intravital imaging methods make it possible to visualize invasive cells *in situ*. Improvement of the spatial and temporal resolution of intravital microscopy will be necessary to visualize the dynamics of cellular components and invasive structures of tumour cells in their native environment *in vivo*. Questions that remain unanswered are what is the role of polarised intracellular trafficking in the formation, extension, and functionality of invasive adhesions/protrusions in matrix remodelling? Second, what do invadopodia look like *in vivo* in a three-dimensional environment, and is it possible to identify them by morphological criteria? Is the current definition of invadopodia in 2D applicable to the 3D situation, and if not, how would it have to be modified?

Implications for anti-angiogenic therapies

This study has provided evidence that specifically increasing levels of HIF-1α either by chemical, over-expression or environmental strategies increases the invasive potential of cancer cells. Such data demonstrating a direct correlation between hypoxic environment and invasive potential has serious consequences for the treatment of solid tumours with anti-angiogenic agents. Anti-angiogenic therapy by way of targeting VEGF pathway has been thought to hold significant potential for

the treatment of cancer (Folkman et al., 1971). However, clinical trials among breast cancer patients reveal only limited effectiveness of anti-angiogenic agents in prolonging patient survival (Folkman, 2007). Recent research using preclinical models further suggests that the administration of anti-angiogenic agents generate intratumoral hypoxia which actually increases invasive and metastatic properties of breast cancer cells, mediated by the HIF-1α pathway (Conley et al., 2012). In contrast, a recent study has indicated that simultaneous inhibition of c-Met and VEGF signalling not only slows tumour growth but also reduces invasion and metastasis (Sennino et al., 2012). This suggests a role for HGF/c-Met pathway in development of resistance to anti-angiogenic therapy and represents a potential strategy to bypass resistance to VEGFR inhibition.

How might tumours become more invasive during anti-angiogenic therapy? One possibility is that tumours may elevate the activity of a pre-existing invasion program that was not previously the driving force of expansive tumour growth, given the capability for angiogenesis. Alternatively, some tumours may switch on an invasive growth program distinct from that arising spontaneously during unperturbed tumour development and progression. These observations suggest that the invasive growth program induced in response to therapy may be qualitatively different than the pathway used in normal tumour progression. The realization that potent angiogenesis inhibition can alter the natural history of tumours by increasing invasion and metastasis warrants clinical investigation, as the prospect has important implications for the development of enduring antiangiogenic therapies. In human tumours, the expression of *MET* correlates with HIF-1α expression (which is usually higher in the inner mass where oxygen diffusion is hampered) (Pennacchietti et al., 2003; Scarpino et al., 2004). HIF-1α can promote cell motility and invasion by inducing c-Met transcription (Hara et al., 2006; Pennacchietti et al., 2003) and sensitizing cells to HGF stimulation (Pennacchietti et al., 2003). A positive feedback loop may contribute to sustain and amplify MET over expression in hypoxic regions of solid tumours since the c-Met pathway induces both the *Met* gene itself (Boccaccio et al., 1994) and HIF-1 activity (Tacchini et al., 2001; Tacchini et al., 2003). In the future it will be important to better understand how hypoxia changes the behaviour of the cancer cell when considering therapeutic approaches.

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APPENDIX

Symbol	Well	AVG ΔC$_t$ (Ct(GOI) - Ave Ct (HKG))		2^-ΔC$_t$		Fold Change	T-TEST	Fold Up- or Down-Regulation
		Test Sample	Control Sample	Test Sample	Control Sample	Test Sample /Control Sample	p value	Test Sample /Control Sample
ACTN1	A01	1.76	2.04	2.9E-01	2.4E-01	1.21	0.276371	1.21
ACTN3	A02	11.91	12.61	2.6E-04	1.6E-04	1.62	0.009594	1.62
ACTN4	A03	1.85	2.14	2.8E-01	2.3E-01	1.22	0.263954	1.22
ACTR2	A04	1.31	1.81	4.0E-01	2.9E-01	1.41	0.006918	1.41
ACTR3	A05	1.34	1.47	3.9E-01	3.6E-01	1.09	0.496009	1.09
AKT1	A06	3.93	3.98	6.6E-02	6.3E-02	1.03	0.695883	1.03
ARF6	A07	1.54	2.11	3.4E-01	2.3E-01	1.48	0.000461	1.48
ARHGDIA	A08	2.45	2.23	1.8E-01	2.1E-01	0.85	0.004745	-1.17
ARHGEF7	A09	3.86	4.84	6.9E-02	3.5E-02	1.96	0.001467	1.96
BAIAP2	A10	6.91	7.75	8.3E-03	4.6E-03	1.79	0.001493	1.79
BCAR1	A11	5.46	5.36	2.3E-02	2.4E-02	0.93	0.000397	-1.08
CAPN1	A12	6.07	6.05	1.5E-02	1.5E-02	0.99	0.995925	-1.01
CAPN2	B01	0.11	0.40	9.3E-01	7.6E-01	1.22	0.016200	1.22
CAV1	B02	0.21	0.66	8.7E-01	6.3E-01	1.37	0.050492	1.37
CDC42	B03	1.08	1.80	4.7E-01	2.9E-01	1.65	0.016355	1.65
CFL1	B04	-1.59	-1.87	3.0E+00	3.7E+00	0.82	0.045061	-1.22
CRK	B05	2.94	3.15	1.3E-01	1.1E-01	1.16	0.187203	1.16
CSF1	B06	1.83	1.75	2.8E-01	3.0E-01	0.95	0.786278	-1.05
CTTN	B07	5.90	6.00	1.7E-02	1.6E-02	1.07	0.437352	1.07
DIAPH1	B08	4.68	5.13	3.9E-02	2.9E-02	1.36	0.185391	1.36
DPP4	B09	10.74	11.32	5.8E-04	3.9E-04	1.49	0.037688	1.49
EGF	B10	7.73	8.62	4.7E-03	2.5E-03	1.86	0.002203	1.86
EGFR	B11	2.81	3.59	1.4E-01	8.3E-02	1.72	0.016685	1.72
ENAH	B12	10.46	10.35	7.1E-04	7.7E-04	0.93	0.718879	-1.08
EZR	C01	0.98	1.22	5.1E-01	4.3E-01	1.18	0.001460	1.18
FAP	C02	8.55	9.12	2.7E-03	1.8E-03	1.49	0.087958	1.49
FGF2	C03	7.24	7.17	6.6E-03	7.0E-03	0.95	0.486860	-1.05
HGF	C04	12.67	13.03	1.5E-04	1.2E-04	1.28	0.369591	1.28
IGF1	C05	11.92	12.16	2.6E-04	2.2E-04	1.18	0.243220	1.18
IGF1R	C06	4.48	4.64	4.5E-02	4.0E-02	1.12	0.476319	1.12
ILK	C07	3.69	3.61	7.8E-02	8.2E-02	0.95	0.676750	-1.06
ITGA4	C08	5.08	5.45	3.0E-02	2.3E-02	1.29	0.281935	1.29
ITGB1	C09	-0.26	-0.07	1.2E+00	1.0E+00	1.14	0.093323	1.14
ITGB2	C10	6.09	6.16	1.5E-02	1.4E-02	1.05	0.934516	1.05
ITGB3	C11	5.06	6.16	3.0E-02	1.4E-02	2.14	0.016352	2.14
LIMK1	C12	4.61	4.42	4.1E-02	4.7E-02	0.87	0.492810	-1.15
MAPK1	D01	3.69	3.58	7.7E-02	8.4E-02	0.92	0.738430	-1.08
MET	D02	2.14	2.86	2.3E-01	1.4E-01	1.65	0.033910	1.65
MMP14	D03	2.59	2.36	1.7E-01	2.0E-01	0.85	0.016178	-1.17
MMP2	D04	7.03	7.50	7.7E-03	5.5E-03	1.39	0.233786	1.39
MMP9	D05	5.89	5.87	1.7E-02	1.7E-02	0.99	0.810845	-1.01
MSN	D06	-0.13	0.22	1.1E+00	8.6E-01	1.27	0.114553	1.27
MYH10	D07	4.35	4.50	4.9E-02	4.4E-02	1.10	0.697104	1.10
MYH9	D08	1.06	1.58	4.8E-01	3.3E-01	1.43	0.037418	1.43
MYL9	D09	3.31	2.13	1.0E-01	2.3E-01	0.44	0.000309	-2.28
MYLK	D10	5.70	4.66	1.9E-02	4.0E-02	0.49	0.067095	-2.05
PAK1	D11	4.94	3.90	3.3E-02	6.7E-02	0.49	0.203755	-2.05
PAK4	D12	5.00	5.26	3.1E-02	2.6E-02	1.20	0.305651	1.20
PFN1	E01	-1.44	-1.50	2.7E+00	2.8E+00	0.97	0.636501	-1.04
PIK3CA	E02	4.00	3.83	6.2E-02	7.0E-02	0.89	0.562075	-1.13
PLAUR	E03	0.41	1.95	7.5E-01	2.6E-01	2.90	0.000737	2.90
PLCG1	E04	4.99	4.68	3.2E-02	3.9E-02	0.81	0.251637	-1.24
PLD1	E05	6.16	6.04	1.4E-02	1.5E-02	0.92	0.611809	-1.09
PRKCA	E06	4.31	4.35	5.0E-02	4.9E-02	1.02	0.956182	1.02
PTEN	E07	2.51	2.58	1.8E-01	1.7E-01	1.05	0.973415	1.05
PTK2	E08	2.79	3.31	1.4E-01	1.0E-01	1.43	0.299814	1.43
PTK2B	E09	7.44	7.24	5.8E-03	6.6E-03	0.87	0.555507	-1.15
PTPN1	E10	3.63	2.75	8.1E-02	1.5E-01	0.54	0.036235	-1.84
PXN	E11	2.15	2.39	2.3E-01	1.9E-01	1.18	0.197938	1.18
RAC1	E12	0.13	0.03	9.1E-01	9.8E-01	0.94	0.453375	-1.07
RAC2	F01	3.51	3.24	8.8E-02	1.1E-01	0.83	0.514015	-1.20
RASA1	F02	2.57	2.89	1.7E-01	1.3E-01	1.25	0.646520	1.25
RDX	F03	3.90	3.79	6.7E-02	7.3E-02	0.92	0.683627	-1.08
RHO	F04	10.44	11.32	7.2E-04	3.9E-04	1.84	0.019255	1.84
RHOA	F05	-0.01	-0.08	1.0E+00	1.1E+00	0.95	0.178589	-1.05
RHOB	F06	2.67	2.69	1.6E-01	1.6E-01	1.01	0.981426	1.01
RHOC	F07	1.42	1.45	3.7E-01	3.7E-01	1.02	0.795575	1.02
RND3	F08	2.68	2.28	1.6E-01	2.1E-01	0.75	0.251663	-1.33
ROCK1	F09	6.26	6.13	1.3E-02	1.4E-02	0.91	0.566919	-1.10
SH3PXD2A	F10	5.32	5.25	2.5E-02	2.6E-02	0.95	0.778565	-1.05
SRC	F11	6.19	6.13	1.4E-02	1.4E-02	0.96	0.610721	-1.04
STAT3	F12	2.71	2.70	1.5E-01	1.5E-01	0.99	0.853448	-1.01
SVIL	G01	4.86	4.67	3.4E-02	3.9E-02	0.87	0.575728	-1.15
TGFB1	G02	2.30	3.17	2.0E-01	1.1E-01	1.82	0.051426	1.82
TIMP2	G03	1.75	1.69	3.0E-01	3.1E-01	0.96	0.734263	-1.04
TLN1	G04	2.35	2.67	2.0E-01	1.6E-01	1.25	0.258196	1.25
VASP	G05	5.54	6.55	2.2E-02	1.1E-02	2.02	0.007259	2.02
VCL	G06	2.73	2.48	1.5E-01	1.8E-01	0.84	0.413964	-1.19
VEGFA	G07	-0.03	2.54	1.0E+00	1.7E-01	5.91	0.001374	5.91
VIM	G08	-2.75	-3.07	6.7E+00	8.4E+00	0.80	0.107016	-1.25
WASF1	G09	4.29	4.60	5.1E-02	4.1E-02	1.23	0.577541	1.23
WASF2	G10	2.45	3.28	1.8E-01	1.0E-01	1.78	0.001317	1.78
WASL	G11	7.08	7.53	7.4E-03	5.4E-03	1.36	0.298065	1.36
WIPF1	G12	5.58	11.96	2.1E-02	2.5E-04	83.21	0.035535	83.21
B2M	H01	-0.74	-0.60	1.7E+00	1.5E+00	1.10	0.294318	1.10
HPRT1	H02	3.58	3.84	8.4E-02	7.0E-02	1.20	0.176251	1.20
RPL13A	H03	-0.20	-0.20	1.1E+00	1.2E+00	1.00	0.839547	-1.00
GAPDH	H04	-2.68	-2.86	6.4E+00	7.3E+00	0.88	0.198335	-1.13
ACTB	H05	-2.64	-3.04	6.2E+00	8.2E+00	0.76	0.006593	-1.32